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# Temperature sensitivity of substrate-use efficiency can result from altered microbial physiology without change to community composition

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1 **Temperature sensitivity of substrate-use efficiency can result from altered microbial**  
2 **physiology without change to community composition**

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17  
18 *Keywords*: carbon-use efficiency; thermodynamic efficiency; microbial community  
19 composition; temperature sensitivity; substrate quality; isothermal calorimetry

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26 **Highlights**

- 27 • Substrate-use efficiency decreased significantly with higher incubation temperatures
- 28 • Minor shifts in active microbial communities were observed across temperatures
- 29 • Shifts in community composition cannot explain large decreases in efficiency
- 30 • Microbial physiology is primarily responsible for decreases in efficiency

31

32 **Abstract**

33 Mechanisms controlling carbon stabilisation in soil and its feedback to climate change are of  
34 considerable importance. Microbial substrate-use efficiency is an important property during  
35 decomposition of soil organic matter. It determines the allocation of substrate towards  
36 biosynthetic stabilisation of carbon and for respiratory losses into the atmosphere. Previously,  
37 it was observed that substrate-use efficiency declines with an increase in temperature and that  
38 it varies across organic substrates. Yet, our mechanistic understanding of processes causing  
39 the temperature sensitivity of substrate-use efficiency is limited. Changes in substrate-use  
40 efficiency could be triggered by (i) shifts in the active components of microbial communities,  
41 (ii) changes in microbial physiology within the same community, or (iii) a combination of  
42 both. In the present study, we evaluated the link between microbial community composition  
43 and substrate-use efficiency, combining measurements of carbon mineralisation and  
44 microbial energetics. We found only minor shifts in microbial community composition,  
45 despite large differences in substrate-use efficiencies across incubation temperatures and  
46 substrate additions. We conclude that short-term changes in substrate-use efficiency were  
47 mainly caused by changes in microbial physiology, but emphasize that future studies should  
48 focus on resolving long-term trade-offs between physiological and community influences on  
49 substrate-use efficiency.

## 50 **1. Introduction**

51 Global soil carbon (C) stocks exceed atmospheric C by more than three times and above-  
52 ground biomass C by more than four times (Ciais et al., 2013). In light of predicted climate  
53 change, the balance between soil C sequestration and soil organic C decomposition is of  
54 considerable importance (e.g. Allison et al., 2010; Davidson and Janssens, 2006; Melillo,  
55 2002). During microbial decomposition of soil organic matter (SOM), organic C is allocated  
56 for respiratory energy production and towards biosynthetic stabilisation such as cell  
57 maintenance and growth. This partitioning (microbial substrate-use efficiency) is often  
58 assumed to be a constant in process-based models (e.g. Coleman and Jenkinson, 1996; Parton  
59 et al., 1987). Some studies (e.g. Dijkstra et al., 2011; Frey et al., 2013; Hagerly et al., 2014)  
60 confirm this assumption when glucose was used as a substrate across various incubation  
61 temperatures. In contrast, studies using a range of substrates (e.g. Devêvre and Horwáth,  
62 2000; Frey et al., 2013; Steinweg et al., 2008) and theoretical models (Manzoni et al., 2012;  
63 Sinsabaugh et al., 2013) suggest that substrate-use efficiency is temperature dependent,  
64 decreasing with increasing temperature. In theory, the decrease is caused by differing  
65 temperature sensitivities of microbial respiration and substrate uptake (Manzoni et al., 2012).  
66 Still, our mechanistic understanding of the underlying processes is limited. Changes in  
67 substrate-use efficiency with temperature may be caused by (i) physiological changes within  
68 the microbial community actively processing substrates, (ii) shifts in the composition of the  
69 active community, or (iii) a combination of both (Manzoni et al., 2012; Schimel and  
70 Schaeffer, 2012).

71 Microbial substrate-use efficiency is a critical physiological property in the terrestrial C  
72 cycle. At the microbial community level, it could be affected by functional traits and life  
73 history strategies (Schimel and Schaeffer, 2012). Recently, positive correlations were  
74 observed between efficiency and the abundance of Gram-negative bacteria (Bölscher et al.,  
75 2016; Harris et al., 2012) indicating that they may play an important role for varying  
76 substrate-use efficiencies across communities. Furthermore, fungi are commonly assumed to  
77 utilise substrate with higher efficiency than bacteria (e.g. Holland and Coleman, 1987;  
78 Ohtonen et al., 1999). This idea has been around for some time, but although some studies  
79 support this notion (Bölscher et al., 2016; Kallenbach et al., 2016), others do not (Brant et al.,  
80 2006; Thiet et al., 2006). Seasonal dynamics in microbial community composition, with a  
81 shift towards increased relative abundance of fungi in winter, have been observed  
82 (Buckeridge et al., 2013; Schadt et al., 2003). Such a shift towards relatively more active

83 fungi at lower temperature may be an explanation in observed increase in efficiencies with  
84 lower temperatures. Yet, few studies complemented direct measurements of substrate-use  
85 efficiency across temperatures with investigations of community composition.

86 Estimates of microbial substrate-use efficiency often rely on quantification of  $^{13}\text{C}$  in  
87 microbial biomass after uptake of labelled substrate. The fumigation-extraction method  
88 (Vance et al., 1987) is commonly used for this evaluation, but may not be wholly reliable  
89 shortly after substrate addition (Marstorp and Witter, 1999; Witter and Dahlin, 1995).

90 Recently, we proposed a microbial energetics approach using isothermal calorimetry to  
91 quantify substrate-use efficiency (Bölscher et al., 2016; Harris et al., 2012). We suggested the  
92 determination of thermodynamic efficiency by adapting the equations of Battley (1987,  
93 1960). This approach does not rely on measurements of substrate incorporation into microbial  
94 biomass by fumigation-extraction. Microbial energetics approaches quantify net metabolic  
95 heat production which covers all metabolic processes and not only those leading to  $\text{CO}_2$   
96 production making them complementary to respiratory-based investigations (Herrmann et al.,  
97 2014). Here, we combine energetics and respiratory approaches to compare results from both.

98 The aim of the present study was to investigate links between changes in microbial  
99 community composition and temperature sensitivity of substrate-use efficiency. The  
100 hypothesis in the present study was that temperature sensitive microbial substrate-use  
101 efficiencies are linked to shifts in the composition of the active microbial community. To test  
102 this hypothesis, we analysed substrate-use efficiency in two contrasting soil treatments at two  
103 temperatures by complementing substrate induced respiration using isotopic  $^{13}\text{C}$ -labelling  
104 with microbial energetics determined via isothermal calorimetry. A set of substrates was used  
105 as previous research has shown that temperature sensitivity of microbial substrate-use  
106 efficiency is substrate dependent (Frey et al., 2013).

## 107 **2. Material and Methods**

### 108 *2.1. Soils*

109 Soil samples were collected at the Pilmore soil cultivation experiment ( $56^{\circ}27'\text{N}$ ,  $03^{\circ}04'\text{W}$ ,  
110 the James Hutton Institute, Dundee, UK), on a freely-drained Dystric-Fluvis Cambisol  
111 (IUSS, 2006) with a sandy-loam texture (Griffiths et al., 2010). The site was established in  
112 2005 and was exposed to a maritime climate with an average annual air temperature of  $8.3^{\circ}\text{C}$   
113 ( $12^{\circ}\text{C}$  at time of sampling) and precipitation of 720 mm (30 year average, Ghee et al., 2013).  
114 Additional soil properties are given in Table 1. Soil treatments consisted of different organic

115 amendments in three split-plot randomised field replicates. Sampled plots received either  
116 (i) compost amendments at an annual rate of 35 t/ha (Compost) or (ii) cropped control plots  
117 were without organic amendments (Unamended). Spring barley is grown annually on all  
118 plots. Soil was collected from 5 – 15 cm depths after removal of barley stubbles in October  
119 2014. Samples from three field replicates were pooled into one composite sample to achieve  
120 samples that were representative of field treatments while minimising influences of plot  
121 variation. The study focused on fundamental understanding of temperature effects on  
122 substrate-use efficiency and therefore small variation among samples was of importance.  
123 Samples were sieved to 2 mm, visible plant fragments and macro fauna removed, and water  
124 contents were adjusted to 45 % water holding capacity. The samples were divided into sub-  
125 sets and frozen until further analysis to suppress microbial activity as all measurements could  
126 not be carried out simultaneously. One sub-set was used to investigate C mineralisation,  
127 another for determination of microbial energetics. For each experimental treatment, three  
128 laboratory replicates were used from each composite sample.

## 129 *2.2. Incubation experiments*

### 130 2.2.1. Carbon mineralisation

131 Soil samples were thawed and incubated at 20 °C for two days to allow the initial respiratory  
132 flush upon freeze-thawing to subside (Herrmann and Witter, 2002). The samples were then  
133 pre-incubated at either 5 or 20 °C for ten days. The 10-day pre-incubation was based on  
134 previous observations (Cookson et al., 2007) indicating that short-term time intervals should  
135 be sufficient for altering microbial community composition at temperatures similar to the  
136 present study.

137 For each temperature, field treatment and laboratory replicate, aliquots (30 g dry weight)  
138 were weighed into plastic jars, pre-incubated and soils then received 4.16  $\mu\text{mol C g}^{-1}$  soil of  
139 either D-glucose, D-trehalose, L-alanine, or phenol. Control samples received ultrapure  
140 water. Substrate solutions contained a mixture of unlabelled and 99 atom %  $^{13}\text{C}$  uniformly-  
141 labelled substrate resulting in a final enrichment of 4.9 to 5.5 atom-%  $^{13}\text{C}$ . Substrate additions  
142 adjusted the water content of soils to approx. 55 % water holding capacity. Samples were  
143 placed into gastight 0.58 l Kilner jars and headspaces were purged with  $\text{CO}_2$  free air until the  
144  $\text{CO}_2$  concentration was below 4 ppm. The soils were incubated for 24 h at the experimental  
145 temperatures of 5 or 20 °C and headspace gas samples were taken after 6, 12 and 24 h and  
146 analysed as described below. After each sampling, the headspace was again purged with  $\text{CO}_2$

147 free air. At the end of the incubation period, soil samples were thoroughly homogenised and  
 148 split for analysis of microbial biomass C and phospholipid fatty acid (PLFA) profiles (see  
 149 below).

### 150 2.2.2. Microbial energetics

151 Heat production from substrate utilisation was measured in a parallel experiment, maintaining  
 152 experimental conditions as described above. Replicate soil aliquots (5 g dry weight) were  
 153 placed into 20 ml glass reaction vials and vials were sealed with an admix ampule system  
 154 (TA Instruments, New Castle, DE, USA; Bölscher et al., 2016). Each admix ampule  
 155 contained either D-glucose, D-trehalose, L-alanine, phenol or ultrapure water as control. The  
 156 samples were introduced into a TAM Air isothermal calorimeter (TA Instruments, New  
 157 Castle, DE, USA) set to 5 or 20 °C. After an equilibration time of 7 and 4 hours (5 and 20 °C,  
 158 respectively), substrate solutions were added dropwise, providing 4.16 µmol C g<sup>-1</sup> soil which  
 159 increased the water content to approx. 55 % water holding capacity. Thermal energy  
 160 production rates were recorded continuously over 24 h. At the end of the incubation,  
 161 concentration of CO<sub>2</sub> in the headspace was analysed by gas chromatography (Clarus 500 GC,  
 162 Perkin Elmer Arnel, Waltham, MA, USA) to ensure that the two parallel incubation  
 163 experiments were consistent, allowing comparison of results. Potential abiotic heat  
 164 production from substrate interactions with soil physical properties were evaluated on  
 165 sterilised gamma-irradiated soil samples (25 kGy; CODAN Steritex APS, Espergaerde,  
 166 Denmark; Herrmann et al., 2014).

### 167 2.3. Microbial substrate-use efficiency

168 Measurements of C mineralisation and microbial energetics enabled us to determine  
 169 microbial substrate-use efficiency in units of C as well as energy. Therefore, we investigated  
 170 two efficiency indices (i) carbon-use efficiency (*CUE*) and (ii) thermodynamic efficiency. In  
 171 the following, these two indices refer to the approaches measuring the efficiency of microbial  
 172 substrate use in units of C and energy, respectively. The general term substrate-use efficiency  
 173 is used when both indices revealed consistent trends.

174 Carbon-use efficiency was calculated as described by Frey et al. (2001):

$$175 \quad CUE = \frac{dMBC_{substrate}}{(dMBC_{substrate} + \sum CO_2 - C_{substrate})} \quad (1)$$

176 where  $dMBC_{substrate}$  ( $\mu\text{g C g}^{-1}$  soil) is the change in microbial biomass C from incorporation of  
 177 substrate C and  $\sum CO_2-C_{substrate}$  ( $\mu\text{g C g}^{-1}$  soil) is the cumulative loss of substrate C through  
 178 respiration.

179 The thermodynamic efficiency ( $\eta_{eff}$ ) was calculated using the equation proposed by Bölscher  
 180 *et al.* (2016):

$$181 \quad \eta_{eff} = 1 - \left( \frac{Q_{substrate}}{\Delta H_{initial} - \Delta H_{residual}} \right) \quad (2)$$

182 where  $Q_{substrate}$  ( $\text{J g}^{-1}$  soil) is the cumulative heat production from utilisation of added  
 183 substrate (Eq. 3), and  $\Delta H_{initial}$  and  $\Delta H_{residual}$  ( $\text{J g}^{-1}$  soil) are the combustion enthalpies of the  
 184 substrate initially added and the residual substrate in soil solution at the end of the incubation,  
 185 respectively. The numerator in Eq. (2) is the energy content of substrate undergoing  
 186 decomposition during the incubation period. The enthalpy of combustion  $\Delta H_c$  is 2,816.8 kJ  
 187  $\text{mol}^{-1}$  for D-glucose, 5,648.3 kJ  $\text{mol}^{-1}$  for D-trehalose, 1,626.1 kJ  $\text{mol}^{-1}$  for L-alanine, and  
 188 3,063.8 kJ  $\text{mol}^{-1}$  for phenol (Washburn, 2003). We employed  $\Delta H_{initial}$  values of 1.95 J  $\text{g}^{-1}$  soil  
 189 for D-glucose and D-trehalose, 2.26 J  $\text{g}^{-1}$  soil for L-alanine, and 2.20 J  $\text{g}^{-1}$  soil for phenol. The  
 190 combustion enthalpy of the residual substrate ( $\Delta H_{residual}$ ) was calculated from the substrate  
 191 specific enthalpy of combustion ( $\Delta H_c$ ) and the residual substrate in soil solution ( $DOC_{substrate}$ )  
 192 determined during fumigation-extraction (see below). The cumulative heat production from  
 193 utilisation of added substrates was calculated as follows:

$$194 \quad Q_{substrate} = Q_{treatment} - Q_{control} - Q_{priming} \quad (3)$$

195 where  $Q_{treatment}$  and  $Q_{control}$  ( $\text{J g}^{-1}$  soil) are the heat production from soils with substrate  
 196 addition as well as control samples receiving water only.  $Q_{priming}$  ( $\text{J g}^{-1}$  soil) is the heat  
 197 dissipation in association with priming of soil organic matter mineralisation. It was calculated  
 198 by converting the amount of  $CO_2-C$  released due to priming (see below 2.4.1 Soil respiration)  
 199 to  $\text{J g}^{-1}$  soil using the combustion enthalpy of SOM. The latter was determined on 0.5 g air  
 200 dried soil samples mixed with 1.0 g benzoic acid (standard for calorimetry analysis) using a  
 201 bomb calorimeter (6300 Automatic Isoperibol Calorimeter, Parr Instrument Co., Moline IL,  
 202 USA). Anabolic reactions release or consume only minor heat (Hansen *et al.*, 2004; Sparling,  
 203 1981a, 1981b) and are therefore not considered explicitly when estimating priming effects.

## 204 2.4. Analytical methods

### 205 2.4.1. Soil respiration



206 At each sampling, 12 ml air were taken from the headspace and injected into an IR gas  
 207 analyser (EGM-4, PP systems, Amesbury, MA, USA) for analysis of total CO<sub>2</sub> concentration.  
 208 Another 12 ml headspace sample was injected into a gas sampling vial (Exetainer, Labco  
 209 Limited, Lampeter, UK) for analysis of C isotope ratios (GasBench II interfaced to a Delta  
 210 Plus<sup>XP</sup> IRMS, Thermo Finnigan, Bremen, Germany).

211 The percentage of CO<sub>2</sub>-C derived from the added substrates was calculated using the model  
 212 of Waldrop and Firestone (2004):

$$213 \quad \%C_{substrate} = \left[ \frac{(\delta^{13}C_{control} - \delta^{13}C_{treatment})}{(\delta^{13}C_{control} - \delta^{13}C_{substrate})} \right] * 100 \quad (4)$$

214 where  $\delta^{13}C_{control}$  and  $\delta^{13}C_{treatment}$  are the mean  $\delta^{13}C$  value of the respired CO<sub>2</sub> from control  
 215 and substrate receiving samples, respectively, and  $\delta^{13}C_{substrate}$  is the  $\delta^{13}C$  value of the labelled  
 216 substrate. The absolute CO<sub>2</sub>-C produced from substrate utilisation ( $\sum CO_2-C_{substrate}$ ) was  
 217 calculated from the  $\%C_{substrate}$  and the total, cumulative CO<sub>2</sub>-C respiration. The contribution  
 218 of priming to the soil respiration was determined as increase of CO<sub>2</sub>-C production after  
 219 substrate additions minus the CO<sub>2</sub>-C from substrate utilisation ( $\sum CO_2-C_{substrate}$ ).

#### 220 2.4.2. Microbial biomass and residual substrate

221 Microbial biomass and incorporation of substrate <sup>13</sup>C into biomass were determined using the  
 222 fumigation-extraction method as described by Vance et al. (1987). Organic C content in the  
 223 fumigated and unfumigated soil extracts (0.5 M K<sub>2</sub>SO<sub>4</sub>) was analysed (TOC Analyser model  
 224 700, Corporation, College Station, TX, USA) and C rendered extractable by fumigation was  
 225 converted to microbial biomass C using a *k<sub>ec</sub>* factor of 0.45 (Wu et al., 1990).

226 The signature of <sup>13</sup>C in the microbial biomass was determined from the isotopic signature in  
 227 the soil extracts. Between 0.5 and 2.5 ml extracts were transferred into 12 ml Exetainer<sup>®</sup>  
 228 (Labco Limited, Lampeter, UK) providing 20-60 µg C per sample, and the volume was made  
 229 up to 4 ml with ultrapure water. Dissolved inorganic C was removed by adding 100 µl 1.3 M  
 230 H<sub>3</sub>PO<sub>4</sub>, and subsequently Exetainers were flush-filled with N<sub>2</sub>. Dissolved organic C was  
 231 transferred into the gas phase by adding 100 µl 1.05 M Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and heating the samples for  
 232 30 min at 90 °C. Headspace samples containing the evolved CO<sub>2</sub> were analysed by isotope  
 233 ratio mass spectrometry (GasBench II interfaced to a Delta Plus<sup>XP</sup> IRMS, Thermo Finnigan,  
 234 Bremen, Germany). The  $\delta^{13}C$  values of the microbial biomass were calculated using the  
 235 following equation (Brant et al., 2006):

$$\delta^{13}C_{MBC} = \frac{(\delta^{13}C_{fum} * C_{fum} - \delta^{13}C_{unfum} * C_{unfum})}{(C_{fum} - C_{unfum})} \quad (5)$$

where  $\delta^{13}C_{fum}$  and  $\delta^{13}C_{unfum}$  are the  $\delta^{13}C$  values of the fumigated and unfumigated samples, respectively, and  $C_{fum}$  and  $C_{unfum}$  are the amounts of C extracted from these samples, respectively.  $\delta^{13}C_{MBC}$  values were used to calculate changes in microbial biomass C from incorporation of substrate C ( $dMBC_{substrate}$ ), the latter is required for Eq. (1). Therefore, the percentage of microbial biomass coming from the utilisation of added substrate was calculated using Eq. (4). Here,  $\delta^{13}C_{treatment}$  was replaced with  $\delta^{13}C_{MBC}$  assuming that the  $\delta^{13}C$  of the control samples reflect the microbial biomass C at the start of the experiment. The residual substrate in soil solution after 24 hours ( $DOC_{substrate}$  used in Eq. 2) was calculated from the dissolved organic C (DOC) in the unfumigated substrate treatments and control samples using Eq. (4).

#### 2.4.3. Microbial community composition

Microbial community composition and incorporation of substrate  $^{13}C$  into microbial groups was analysed by phospholipid fatty acid composition (PLFA) (Frostegård et al., 1993). PLFA abundance and  $^{13}C$  isotopic enrichment were determined via GC-C-IRMS (TraceUltra GC interfaced to Delta<sup>Plus</sup> XP IRMS via GC Combustion III, Thermo Finnigan, Bremen, Germany). Isotope ratios were corrected for C added during the PLFA extraction procedure using a mass-balance equation (Crossman et al., 2004). The abundance of specific microbial groups was evaluated by assigning PLFA biomarkers to these groups as follows: Gram-positive i14:0, i15:0, a15:0, i16:0, i17:0, a17:0; Gram-negative 16:1 $\omega$ 7, cy17:0, 18:1 $\omega$ 7, cy19:0; actinomycetes 10Me16:0, 10Me17:0, 10Me18:0; and fungi 18:2 $\omega$ 6,9 (Bååth, 2003; Frostegård and Bååth, 1996; Zelles, 1999).

Substrate C incorporation into biomarkers of specific microbial groups was expressed as (i) relative incorporation and (ii) specific incorporation of substrates into biomarkers:

$$^{13}C_{relative\ incorp.} = \frac{^{13}C_{biomarker}}{^{13}C_{PLFA}} \quad (6)$$

$$^{13}C_{specific\ incorp.} = \frac{^{13}C_{relative\ incorp.}}{C_{biomarker}} \quad (7)$$

where  $^{13}C_{relative\ incorp.}$  and  $^{13}C_{specific\ incorp.}$  are the relative and specific incorporation, respectively,  $^{13}C_{biomarker}$  is substrate  $^{13}C$  incorporated into biomarkers of a specific microbial group,  $^{13}C_{PLFA}$  is the amount of substrate  $^{13}C$  incorporated into total PLFA, and  $C_{biomarker}$  is

265 the normalised abundance of biomarker C from a specific microbial group. The relative  
266 incorporation provides information about the contribution of a specific microbial group to the  
267 total substrate utilisation. The specific incorporation gives information about the activity of a  
268 specific microbial group during substrate utilisation in relation to its normalised abundance.  
269 Values below or above one indicate that a microbial group is utilising substrate  
270 proportionally less or more actively in relation to its contribution to total PLFA abundance.

271 To test for changes in PLFA profiles due to physiological alteration, i.e. changes in  
272 membrane fluidity, within same communities rather than shifts in community composition  
273 (Schimel et al., 2007; Wixon and Balsler, 2013), the unsaturation index (Ruess et al., 2007),  
274 the monounsaturated-to-saturated ratio as well as the cyclopropyl-to-precursor ratio (Wixon  
275 and Balsler, 2013) of fatty acids were determined in control samples.

## 276 *2.5. Statistics and data analysis*

277 Data were analysed using general linear models with soil, incubation temperature, and  
278 substrate addition as explanatory variables (Minitab 17 Statistical Software, 2010). Data were  
279 tested for normality and equal variance by applying Anderson-Darling test and Leven's test,  
280 respectively. If necessary, data were log-transformed prior to analysis. Homogeneous groups  
281 of means were established using Tukey's HSD test. Within each substrate addition, results  
282 were analysed by one-way ANOVA followed by Tukey's HSD test, and the Student's t-test  
283 was used to evaluate temperature effects for each soil and substrate.

## 284 **3. Results**

### 285 *3.1. Substrate utilisation*

286 CO<sub>2</sub> respiration and heat production were more temperature sensitive than microbial substrate  
287 assimilation (Table 2). Samples incubated at 20 °C released 2 – 20 times more substrate C as  
288 CO<sub>2</sub> and heat than samples incubated at 5 °C ( $P < 0.01$ , respectively; Table 2). In contrast,  
289 incorporation of substrate into biomass was lower at 20 °C than at 5 °C, but differences  
290 between temperatures were significant only for the trehalose treatment in Compost amended  
291 soils (Table 2). Within each temperature, soil respiration and heat production was similar  
292 between Compost and Unamended soils ( $P = 0.48$  and  $P = 0.08$ , respectively; Table 2).  
293 Among substrates, cumulative CO<sub>2</sub> respiration and heat production was highest for alanine  
294 followed by glucose and trehalose additions; CO<sub>2</sub> and heat were released least when phenol  
295 was added to soils ( $P < 0.01$ , respectively; Table 2). At the end of the incubation,

296 concentration of residual C from glucose and trehalose were low in soil solution in both  
297 temperature treatments. In contrast to this, 50 % of added C from alanine was recovered  
298 when samples were incubated at 5 °C and no residual C from alanine was detected at 20 °C  
299 (Table 2). Also, more residual C from phenol was recovered at 5 °C than at 20 °C (Table 2).  
300 Abiotic substrate interactions were negligible as sterilised soils did not induce significant  
301 amounts of heat, and CO<sub>2</sub> mineralisation from the two separate incubations were similar (data  
302 not shown). Priming of soil organic matter mineralisation was minor and there was no  
303 distinct pattern among soils (Table 2). The combustion enthalpy of SOM was significantly  
304 larger for Compost in comparison with the Unamended soil ( $P < 0.01$ , Table 1), but when  
305 expressed as energy per gram of soil organic C than both soils contained approximately 35 kJ  
306 g<sup>-1</sup> soil organic C.

### 307 *3.2. Substrate-use efficiency*

308 Carbon-use efficiency and thermodynamic efficiency revealed overall similar patterns,  
309 though carbon-use efficiencies tended towards values up to 0.08 units higher (Fig. 1).  
310 Differences between the two efficiency approaches were however insignificant as they  
311 correlated linearly (Pearson correlation coefficient = 0.90,  $P < 0.01$ ). For all substrates,  
312 carbon-use and thermodynamic efficiencies were higher when soils were exposed to the  
313 lower incubation temperature of 5 °C in comparison to 20 °C ( $P < 0.01$ , respectively; Fig. 1).  
314 Between soil treatments, microbial communities in Compost amended soils had similar  
315 carbon-use efficiencies in comparison to Unamended soil ( $P = 0.54$ ), whereas thermodynamic  
316 efficiencies were lower in Compost amended soil when soils were incubated at 5 °C  
317 ( $P < 0.01$ , Fig. 1). Furthermore, efficiencies of microbial communities were decreasing in the  
318 following order: phenol > trehalose > glucose > alanine ( $P < 0.01$ , Fig. 1), irrespective of the  
319 index measured. Carbon-use efficiencies for phenol utilisation could not be calculated as  
320 unfumigated samples had larger  $\delta^{13}\text{C}$  values than fumigated soil extracts, and therefore <sup>13</sup>C  
321 incorporation into biomass could not be evaluated in soils receiving phenol (data not shown).

### 322 *3.3. Microbial community composition*

323 Comparing the two soil treatments, relative abundance of microbial groups were as follows:  
324 fungi were more abundant in Unamended soils ( $P < 0.05$ ), Gram-positive bacteria were more  
325 abundant in Compost soils ( $P < 0.01$ ), and Gram-negative bacteria and actinomycetes were  
326 similarly abundant in both soils ( $P = 0.42$  and  $P = 0.17$ , respectively; Table 3). Across  
327 temperature, only the abundance of fungi was altered with more fungal PLFA present at 5 °C

328 in comparison with 20 °C ( $P < 0.01$ , Table 3). In the Compost treatment, both the  
329 unsaturation index and the monounsaturated-to-saturated ratio were greater at 5 °C in  
330 comparison to 20 °C incubation temperature ( $P < 0.05$ , respectively; Table 3). Similar trends  
331 were observed in the Unamended soil samples although the differences were not significant  
332 ( $P = 0.43$  and  $0.41$ , respectively; Table 3). The cyclopropyl-to-precursor ratios increased with  
333 temperature in both soil treatments but results were not significant ( $P = 0.37$  and  $0.36$ ,  
334 respectively; Table 3).

335 The addition of  $^{13}\text{C}$  substrates did not alter the composition of PLFA profiles, except for soils  
336 receiving phenol, here, the relative abundance of fungi increased (data not shown). However,  
337 the abundance of the fungal biomarker 18:2 $\omega$ 6,9 was low and often below the detection limit,  
338 i.e. no biomarker was detected in 15 out of 60 samples. Proportionally, the highest amount of  
339 substrate was incorporated into unspecific lipids followed by Gram-negative bacteria, Gram-  
340 positive bacteria, actinomycetes and fungi incorporated least  $^{13}\text{C}$  substrates (Fig. 2). This  
341 general pattern reflects the relative abundance of PLFA (Table 3). With increasing  
342 temperature, relative substrate incorporation was increased in Gram-positive bacteria and  
343 relatively less substrate was incorporated into Gram-negative bacteria ( $P < 0.01$  and  $P < 0.05$ ,  
344 respectively; Fig. 2 A-D). Substrate incorporation was similar for actinomycetes, fungi and  
345 unspecific lipids across temperatures ( $P = 0.17$ ,  $0.07$ , and  $0.34$ ; respectively). Relative  
346 substrate incorporation among the various substrates and within biomarker groups revealed  
347 the following pattern: Gram-negative bacteria incorporated more alanine, Gram-positive  
348 bacteria assimilated less and fungi more phenol, and more trehalose as well as phenol were  
349 incorporated into unspecific lipids ( $P < 0.01$ , respectively; Fig. 2 A-D). Although these  
350 differences were significant, changes in substrate incorporation at different temperatures  
351 among microbial biomarkers were small and in absolute values less than 10 %. In relation to  
352 their relative abundance (i.e. specific substrate incorporation), actinomycetes were less active  
353 in utilising added  $^{13}\text{C}$  substrates, i.e. specific substrate incorporation was less than one  
354 (Fig. 2 E-H). Further, Gram-positive bacteria were less and fungi were more active in  
355 utilising phenol (Fig. 2 H), and Gram-negative were more active in utilising alanine  
356 (Fig. 2 G).

## 357 **4. Discussion**

### 358 *4.1. Substrate-use efficiency*

359 Equivalent amounts of substrate-C were mineralised in the two parallel experiments to  
360 quantify soil CO<sub>2</sub> efflux and microbial energetics. Irrespective of substrate additions,  
361 microbial substrate-use efficiency was lower at the higher temperature (Fig. 1) confirming  
362 theoretical models and empirical evidence regarding the temperature dependence of  
363 substrate-use (Frey et al., 2013; Manzoni et al., 2012; Steinweg et al., 2008; Tucker et al.,  
364 2013). Patterns for thermodynamic efficiency and carbon-use efficiency were similar: on  
365 average, efficiencies of microbial communities in soils receiving glucose, trehalose or phenol  
366 were 0.06 – 0.15 units lower when incubated at 20 °C than at 5 °C (Fig. 1). These differences  
367 are similar to previous studies (Frey et al., 2013; Schindlbacher et al., 2015; Steinweg et al.,  
368 2008; Tucker et al., 2013). In soils with alanine addition, effects of temperature on efficiency  
369 were more pronounced and up to 0.53 units lower at 20 °C (Fig. 1). The mechanism behind  
370 this observation is elusive, but the following two aspects may have contributed: (i) alanine is  
371 an amino acid and a source of nitrogen, and (ii) potential bias inherent in the experimental  
372 approaches applied.

373 (i) As alanine is an amino acid and source of nitrogen, it can trigger overflow respiration, due  
374 to excess mineralisation of C for nitrogen gain (Keiblinger et al., 2010; Manzoni et al., 2012;  
375 Schimel and Weintraub, 2003). This may contribute to the more pronounced difference in  
376 efficiency between temperatures in samples receiving alanine compared with other substrates.  
377 On the one hand, microbial activity increases significantly with temperature supporting a  
378 possible overflow respiration in samples receiving alanine at 20 °C. On the other hand,  
379 alanine has a very narrow C-to-nitrogen ratio of three which is below the critical threshold for  
380 biomass and the natural SOM has ratios below thirteen (Table 1). It is therefore less certain  
381 that microorganisms produce overflow respiration as they are not in need for nitrogen gain  
382 (Sinsabaugh et al., 2016, 2013). The lack of substantial priming (Table 2) does also not  
383 support overflow respiration. Our results, however, emphasize that nitrogen may have a  
384 significant impact on temperature sensitivity of substrate-use efficiency, and there is a need to  
385 improve our understanding of the underlying mechanism. Future temperature studies could  
386 compare alanine and pyruvate, a C substrate with a similar structure as alanine except that the  
387 second C-atom has an oxygen instead of an amino-group, and/or using <sup>13</sup>C-<sup>15</sup>N dual-labelled  
388 alanine (Andresen et al., 2009).

389 (ii) The approaches to quantify efficiency differ in how they account for assimilatory  
390 processes (compare Eq. 1 and Eq. 2). Thermodynamic efficiency considers residual substrate,  
391 while carbon-use efficiency considers C incorporation into microbial biomass. Carbon-use

392 efficiencies decreased more strongly with increased temperature in samples receiving alanine  
393 in comparison to thermodynamic efficiencies. In the present study, thermodynamic efficiency  
394 assumes that  $^{13}\text{C}$  recovered in the DOC pool represents only unused added substrate, i.e.  
395 residual substrate. Microbial excretion of recently assimilated  $^{13}\text{C}$  into the DOC pool would  
396 result in an underestimation whereas adsorption of substrate would result in an  
397 overestimation of thermodynamic efficiency. We cannot evaluate if microbial excretion  
398 contributes significantly to the  $^{13}\text{C}$  DOC pool, but it is unlikely that 50 % recovery as residual  
399 substrate at 5 °C after 24 h (Table 2) is mainly due to excretion. Also, we have some  
400 indications that adsorption is unlikely to be a factor introducing an experimental bias: The  
401 recovery was 83 and 92 % of the alanine C added to Compost and Unamended soils  
402 incubated at 5 °C, respectively and  $\text{K}_2\text{SO}_4$  extraction should have recovered alanine C from  
403 the soil matrix. Future research should focus on method development to differentiate residual  
404 substrate from secreted  $^{13}\text{C}$ -compounds, e.g. estimation of metabolites in soil solution using  
405  $^1\text{H}$ -NMR metabolomics (Hwang and Shaka, 1995; Tiziani et al., 2008). The methodological  
406 biases for thermodynamic efficiency mentioned above are also valid for carbon-use  
407 efficiency. In addition, assessment of microbial biomass via fumigation-extraction may be  
408 biased by non-uniform incorporation of substrate-C into microbial biochemical components  
409 in short-term incubation experiments (Marstorp and Witter, 1999; Witter and Dahlin, 1995).  
410 Results from substrate incorporation into biomass showed larger variation than measurements  
411 of residual substrate (Table 2). Therefore, it seems more likely that carbon-use efficiency  
412 values underestimate efficiency in samples receiving alanine due to a poor recovery of C  
413 incorporated into biomass.

414 Substrate-use efficiencies varied in our experiment between soils when different substrates  
415 were added (Fig. 1), which was observed previously (Bölscher et al., 2016; Frey et al., 2013).  
416 Surprisingly, microbial communities utilised phenol with the highest thermodynamic  
417 efficiency of all substrates (Fig. 1), despite phenol's aromatic properties. Such compounds  
418 require initial enzymatic cleavage steps during break-down, resulting in high energetic costs  
419 for enzyme production which would be expected to lead to relatively low substrate-use  
420 efficiencies (Manzoni et al., 2012). Previous studies observed much smaller efficiencies  
421 ranging between 0.2 and 0.4 when adding phenol to soil (Brant et al., 2006; Frey et al., 2013).  
422 Our high values maybe partially be due to methodological biases (see discussion above) as  
423 phenol may have been lost through volatilization or adsorbed onto soil minerals leading to an  
424 overestimation of thermodynamic efficiency (Eq. 2). But it is unlikely that such biases are

425 solely responsible for the observed high efficiency. Very recently, Kallenbach et al. (2016)  
426 observed substrate-use efficiencies of ca. 0.7 when adding syringol, another phenolic  
427 compound to artificial model soils. Their study is based on an indirect approach using  $^{13}\text{C}$   
428 glutamic acid (for detailed method description see Kallenbach et al., 2016) suggesting that  
429 high efficiencies may be feasible when phenolic compounds are added to soils. These  
430 observations warrant further investigations into substrate-use efficiency of aromatic  
431 compounds and their temperature sensitivity.

432 Overall, microbial substrate-use efficiency estimated with both approaches were high and  
433 varied between 0.51-0.98 and 0.37-0.94 for thermodynamic and carbon-use efficiency,  
434 respectively (Fig. 1). Such high values are commonly observed in short-term soil incubation  
435 experiments (Bölscher et al., 2016; Frey et al., 2013; Schindlbacher et al., 2015; Steinweg et  
436 al., 2008), but it is questioned if they are representative for *in situ* performance of microbial  
437 communities in soils (Sinsabaugh et al., 2013; Spohn et al., 2016). The theoretical  
438 thermodynamic maximum values of substrate-use efficiencies for glucose and alanine  
439 (Gommers et al., 1988) are in a range similar to the present study, and Dijkstra et al. (2015)  
440 argued recently that high substrate-use efficiencies may be possible *in situ* in soils. Their  
441 reasoning was that it would only require a minor proportion of the microbial community to  
442 grow and utilise substrate with high efficiency to drive overall efficiency of the whole  
443 community towards high values. Irrespective of questions over high absolute values for  
444 substrate-use efficiency (Sinsabaugh et al., 2013; Spohn et al., 2016), small changes in  
445 efficiencies across temperatures may have large impacts when projecting soil C stocks  
446 (Bölscher, 2016; Hyvönen et al., 1998). We therefore suggest that in the future, temperature  
447 sensitive efficiencies derived from laboratory experiments could be incorporated into  
448 numerical models (Coleman and Jenkinson, 2014; Parton et al., 1987; Ågren and Bosatta,  
449 1998) by using relative rather than absolute changes for substrate-use efficiency.

#### 450 4.2. Microbial community composition

451 The 10-day pre-incubation at different temperatures and the addition of substrates did not  
452 alter the composition of PLFA biomarkers significantly as PLFA-profiles were similar in all  
453 treatments (data not shown). This indicates no significant shifts in microbial community  
454 composition (Brant et al., 2006), e.g. fast growing copiotrophs out-competing slow growing  
455 oligotrophs (Shen and Bartha, 1996) during the incubation experiment. Consequently, we  
456 interpret the observed differences in substrate incorporation as variation in microbial activity



457 during decomposition (Brant et al., 2006) rather than a shift within the microbial community  
458 triggered by the level of substrate addition (Shen and Bartha, 1996). Using <sup>13</sup>C- labelled  
459 substrate allowed us to trace substrate incorporation into various microbial groups, but the  
460 differences in label incorporation within groups were small among sample treatments  
461 (Fig. 2). Although being an important group within soil communities, actinomycetes showed  
462 low activity in using any of the added substrates (Fig. 2), and hence they contributed little to  
463 the substrate-use efficiency of the overall microbial communities in this experiment. They are  
464 therefore not further considered.

465 Relatively more Gram-negative bacteria were active at 5 °C, where efficiencies were  
466 consistently higher (Fig. 2). This observation could support previous findings that Gram-  
467 negative bacteria have high substrate-use efficiencies (Bölscher et al., 2016; Harris et al.,  
468 2012). Gram-negative bacteria also showed higher activities in using alanine. Yet, alanine  
469 was utilised with the lowest efficiency of all substrates, opposing the assumption that Gram-  
470 negative bacteria are relatively efficient. Alanine is, however, a nitrogen source and may have  
471 triggered overflow respiration. Therefore, this finding may indicate a higher nitrogen demand  
472 of Gram-negative bacteria rather than a generally, relatively low substrate-use efficiency.  
473 Findings regarding Gram-negative bacteria were unaffected by possible temperature induced  
474 changes in lipid composition as the group of Gram-negative biomarkers contained precursor  
475 (16:1 $\omega$ 7 and 18:1 $\omega$ 7) and successor (cy17:0 and cy19:0) lipids and its sum would be  
476 unaffected by changes in lipid composition.

477 Gram-positive bacteria were more active in utilising added substrates at 20 °C where  
478 efficiencies were low (Fig. 2) indicating a generally lower efficiency for this microbial group.  
479 The results should be treated with caution as temperature induced physiological alterations of  
480 PLFA profiles may introduce a bias. Gram-positive biomarkers are exclusively saturated fatty  
481 acids and these fatty acids tend to increase with temperature (Wixon and Balsler, 2013). More  
482 abundant Gram-positive biomarkers may therefore be a result of changes in lipid composition  
483 of membranes as a direct response to temperature and not an increase in activity.

484 Nevertheless, the higher activity at 20 °C cannot be explained exclusively by temperature  
485 induced alteration of lipid composition, because the higher activity of Gram-positive bacteria  
486 at 20 °C was also evident in the specific substrate incorporation (Fig. 2 E-H). The latter is  
487 less affected by changes in lipid composition, as it is the ratio of incorporated substrate per  
488 total abundance of a specific biomarker group. Changes in lipid composition should modify  
489 both values proportionally.

490 Fungi were more abundant at 5 °C than at 20 °C (Table 3), supporting the assumption that  
491 fungi are more active at lower temperatures (Buckeridge et al., 2013; Schadt et al., 2003). In  
492 general, fungi incorporated relatively small amounts of glucose, trehalose and alanine (Fig. 2  
493 A-D) indicating low substrate utilization and thus fungi contributed little to efficiency at the  
494 community level. However, the fungal biomarker showed remarkably high label  
495 incorporation in the phenol treatment, in particular when considering the relative low fungal  
496 abundance (Fig. 2 D and H). Together with the high substrate-use efficiency in phenol  
497 treatment, our results support the common assumption of fungi having higher substrate-use  
498 efficiencies than bacteria (Holland and Coleman, 1987; Ohtonen et al., 1999; Six et al.,  
499 2006). The fungal biomarker 18:2 $\omega$ 6,9 is an unsaturated lipid and its abundance could have  
500 decreased in response to the elevated incubation temperatures (Table 3). Yet, the high fungal  
501 activity was found in both temperature treatments and temperature induced alteration of lipid  
502 composition would have implied low fungal activity rather than high activity.

503 Concluding, fungi and Gram-negative bacteria incorporated more substrate C at the lower  
504 temperature and Gram-positive incorporated more substrate C at the higher temperature. Yet,  
505 all differences within biomarker groups were small.

#### 506 *4.3. Can microbial community composition explain variation in substrate-use efficiency?*

507 The demonstrated shifts in active microbial community cannot explain the large variation of  
508 substrate-use efficiency. Substrate-use efficiency decreased by 0.06 – 0.53 units when  
509 comparing 5 °C to 20 °C (Fig. 1), while relative incorporation of substrate C changes by less  
510 than 10 mol-% C within microbial groups (Fig. 2). The relative substrate C incorporation into  
511 group specific PLFA biomarkers does not represent overall activity of a specific microbial  
512 group in utilising added substrates, because group specific markers are not the only one  
513 present in these organisms' membranes, i.e. some changes are hidden in the unspecific  
514 biomarkers. Still, results from substrate C incorporation into biomarkers give hints about the  
515 contribution of microbial groups to substrate utilisation at different temperatures, e.g. activity  
516 of Gram-negative and Gram-positive bacteria varied between temperatures (see discussion  
517 above). In our experiment, the smallest decrease in efficiency was 0.06 units between the two  
518 temperatures. If we assume that this decrease in substrate-use efficiency was solely caused by  
519 shifts in the active members of microbial communities then substantial variations in substrate  
520 utilisation and efficiencies are required as described in the following example. Assuming that  
521 there are only two microbial groups in the soil: Group A and group B (i.e. all microbial

522 groups other than group A). At 5 °C, group A contributes with 50 % to the overall utilisation  
523 of a substrate and has an efficiency which is 0.20 (i.e. 20 %) higher than the efficiency of  
524 group B. If the overall substrate-use efficiency at the community level should decrease by  
525 0.06 units with increasing temperature, group A would need to reduce its contribution to  
526 substrate utilisation to 20 %. This example illustrates that a large shift in active components  
527 of microbial communities combined with large differences in efficiencies between microbial  
528 groups are required to explain even the small changes in efficiency of 0.06 units. In our  
529 study, we observed efficiency differences up to 0.53 units, and consequently, shifts in the  
530 microbial community actively decomposing substrate are unlikely to cause the observed  
531 differences in substrate-use efficiency between the two temperatures. Therefore, changes in  
532 microbial physiology must be mainly responsible. In our experiment changes in microbial  
533 physiology were indicated by the different temperature sensitivities of microbial respiration  
534 and biomass production. Accumulation of CO<sub>2</sub> and heat production increased strongly while  
535 incorporation of substrate into biomass was less temperature sensitive, but with a tendency  
536 that substrate uptake decreased with increasing temperatures (Table 2). Similar differences in  
537 temperature sensitivity of these processes were also demonstrated by Schindlbacher et al.  
538 (2015) and discussed in a review by Manzoni et al. (2012). In summary, microbial respiration  
539 appears to be the main physiological driver for substrate-use efficiency, but small shifts in  
540 active microbial community may have contributed but to a minor extent in the present short-  
541 term incubation.

## 542 **5. Conclusions**

543 In the present short-term study, substrate-use efficiencies varied across treatments and  
544 decreased with incubation temperature. Nevertheless, shifts in composition of active  
545 microbial community were only small and are likely to be insufficient to explain the large  
546 variations in substrate-use efficiency. Instead, we found evidence for physiological changes  
547 within microbial communities causing most of the variation in efficiency. Yet, some of the  
548 smaller variation in microbial community may have contributed to changes in efficiency, and  
549 our evidence supports prior assumption of relatively higher efficiency of Gram-negative  
550 bacteria and fungi. Results derived from thermodynamic and carbon-use efficiency  
551 measurements were comparable. Prolonged exposure to different temperatures are likely to  
552 influence directly and indirectly community composition. Future studies should therefore  
553 focus on resolving potential trade-offs between physiological and community influences on  
554 substrate-use efficiency in the long-term.

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