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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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Highlights

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

Abstract

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

1. Introduction

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

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

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

Estimates of microbial substrate-use efficiency often rely on quantification of ^{13}C in microbial biomass after uptake of labelled substrate. The fumigation-extraction method (Vance et al., 1987) is commonly used for this evaluation, but may not be wholly reliable shortly after substrate addition (Marstorp and Witter, 1999; Witter and Dahlin, 1995). Recently, we proposed a microbial energetics approach using isothermal calorimetry to quantify substrate-use efficiency (Bölscher et al., 2016; Harris et al., 2012). We suggested the determination of thermodynamic efficiency by adapting the equations of Battley (1987, 1960). This approach does not rely on measurements of substrate incorporation into microbial biomass by fumigation-extraction. Microbial energetics approaches quantify net metabolic heat production which covers all metabolic processes and not only those leading to CO_2 production making them complementary to respiratory-based investigations (Herrmann et al., 2014). Here, we combine energetics and respiratory approaches to compare results from both.

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

2. Material and Methods

2.1. Soils

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

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

2.2. Incubation experiments

2.2.1. Carbon mineralisation

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

For each temperature, field treatment and laboratory replicate, aliquots (30 g dry weight) were weighed into plastic jars, pre-incubated and soils then received 4.16 $\mu\text{mol C g}^{-1}$ soil of either D-glucose, D-trehalose, L-alanine, or phenol. Control samples received ultrapure water. Substrate solutions contained a mixture of unlabelled and 99 atom % ^{13}C uniformly-labelled substrate resulting in a final enrichment of 4.9 to 5.5 atom-% ^{13}C . Substrate additions adjusted the water content of soils to approx. 55 % water holding capacity. Samples were placed into gastight 0.58 l Kilner jars and headspaces were purged with CO_2 free air until the CO_2 concentration was below 4 ppm. The soils were incubated for 24 h at the experimental temperatures of 5 or 20 °C and headspace gas samples were taken after 6, 12 and 24 h and analysed as described below. After each sampling, the headspace was again purged with CO_2

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

2.2.2. Microbial energetics

Heat production from substrate utilisation was measured in a parallel experiment, maintaining experimental conditions as described above. Replicate soil aliquots (5 g dry weight) were placed into 20 ml glass reaction vials and vials were sealed with an admix ampule system (TA Instruments, New Castle, DE, USA; Bölscher et al., 2016). Each admix ampule contained either D-glucose, D-trehalose, L-alanine, phenol or ultrapure water as control. The samples were introduced into a TAM Air isothermal calorimeter (TA Instruments, New Castle, DE, USA) set to 5 or 20 °C. After an equilibration time of 7 and 4 hours (5 and 20 °C, respectively), substrate solutions were added dropwise, providing 4.16 µmol C g⁻¹ soil which increased the water content to approx. 55 % water holding capacity. Thermal energy production rates were recorded continuously over 24 h. At the end of the incubation, concentration of CO₂ in the headspace was analysed by gas chromatography (Clarus 500 GC, Perkin Elmer Arnel, Waltham, MA, USA) to ensure that the two parallel incubation experiments were consistent, allowing comparison of results. Potential abiotic heat production from substrate interactions with soil physical properties were evaluated on sterilised gamma-irradiated soil samples (25 kGy; CODAN Steritex APS, Espergaerde, Denmark; Herrmann et al., 2014).

2.3. Microbial substrate-use efficiency

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

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

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

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

The thermodynamic efficiency (η_{eff}) was calculated using the equation proposed by Bölscher *et al.* (2016):

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

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

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

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

2.4. Analytical methods

2.4.1. Soil respiration

At each sampling, 12 ml air were taken from the headspace and injected into an IR gas analyser (EGM-4, PP systems, Amesbury, MA, USA) for analysis of total CO₂ concentration. Another 12 ml headspace sample was injected into a gas sampling vial (Exetainer, Labco Limited, Lampeter, UK) for analysis of C isotope ratios (GasBench II interfaced to a Delta Plus^{XP} IRMS, Thermo Finnigan, Bremen, Germany).

The percentage of CO₂-C derived from the added substrates was calculated using the model of Waldrop and Firestone (2004):

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

where $\delta^{13}C_{\text{control}}$ and $\delta^{13}C_{\text{treatment}}$ are the mean $\delta^{13}C$ value of the respired CO₂ from control and substrate receiving samples, respectively, and $\delta^{13}C_{\text{substrate}}$ is the $\delta^{13}C$ value of the labelled substrate. The absolute CO₂-C produced from substrate utilisation ($\sum \text{CO}_2\text{-C}_{\text{substrate}}$) was calculated from the $\%C_{\text{substrate}}$ and the total, cumulative CO₂-C respiration. The contribution of priming to the soil respiration was determined as increase of CO₂-C production after substrate additions minus the CO₂-C from substrate utilisation ($\sum \text{CO}_2\text{-C}_{\text{substrate}}$).

2.4.2. Microbial biomass and residual substrate

Microbial biomass and incorporation of substrate ¹³C into biomass were determined using the fumigation-extraction method as described by Vance et al. (1987). Organic C content in the fumigated and unfumigated soil extracts (0.5 M K₂SO₄) was analysed (TOC Analyser model 700, Corporation, College Station, TX, USA) and C rendered extractable by fumigation was converted to microbial biomass C using a *k_{ec}* factor of 0.45 (Wu et al., 1990).

The signature of ¹³C in the microbial biomass was determined from the isotopic signature in the soil extracts. Between 0.5 and 2.5 ml extracts were transferred into 12 ml Exetainer[®] (Labco Limited, Lampeter, UK) providing 20-60 µg C per sample, and the volume was made up to 4 ml with ultrapure water. Dissolved inorganic C was removed by adding 100 µl 1.3 M H₃PO₄, and subsequently Exetainers were flush-filled with N₂. Dissolved organic C was transferred into the gas phase by adding 100 µl 1.05 M Na₂S₂O₈ and heating the samples for 30 min at 90 °C. Headspace samples containing the evolved CO₂ were analysed by isotope ratio mass spectrometry (GasBench II interfaced to a Delta Plus^{XP} IRMS, Thermo Finnigan, Bremen, Germany). The $\delta^{13}C$ values of the microbial biomass were calculated using the 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^{Plus} 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 ω 7, cy17:0, 18:1 ω 7, cy19:0; actinomycetes 10Me16:0, 10Me17:0, 10Me18:0; and fungi 18:2 ω 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

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

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

2.5. Statistics and data analysis

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

3. Results

3.1. Substrate utilisation

CO₂ respiration and heat production were more temperature sensitive than microbial substrate assimilation (Table 2). Samples incubated at 20 °C released 2 – 20 times more substrate C as CO₂ and heat than samples incubated at 5 °C ($P < 0.01$, respectively; Table 2). In contrast, incorporation of substrate into biomass was lower at 20 °C than at 5 °C, but differences between temperatures were significant only for the trehalose treatment in Compost amended soils (Table 2). Within each temperature, soil respiration and heat production was similar between Compost and Unamended soils ($P = 0.48$ and $P = 0.08$, respectively; Table 2). Among substrates, cumulative CO₂ respiration and heat production was highest for alanine followed by glucose and trehalose additions; CO₂ and heat were released least when phenol was added to soils ($P < 0.01$, respectively; Table 2). At the end of the incubation,

concentration of residual C from glucose and trehalose were low in soil solution in both temperature treatments. In contrast to this, 50 % of added C from alanine was recovered when samples were incubated at 5 °C and no residual C from alanine was detected at 20 °C (Table 2). Also, more residual C from phenol was recovered at 5 °C than at 20 °C (Table 2). Abiotic substrate interactions were negligible as sterilised soils did not induce significant amounts of heat, and CO₂ mineralisation from the two separate incubations were similar (data not shown). Priming of soil organic matter mineralisation was minor and there was no distinct pattern among soils (Table 2). The combustion enthalpy of SOM was significantly larger for Compost in comparison with the Unamended soil ($P < 0.01$, Table 1), but when expressed as energy per gram of soil organic C than both soils contained approximately 35 kJ g⁻¹ soil organic C.

3.2. Substrate-use efficiency

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

3.3. Microbial community composition

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

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

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

4. Discussion

4.1. Substrate-use efficiency

Equivalent amounts of substrate-C were mineralised in the two parallel experiments to quantify soil CO₂ efflux and microbial energetics. Irrespective of substrate additions, microbial substrate-use efficiency was lower at the higher temperature (Fig. 1) confirming theoretical models and empirical evidence regarding the temperature dependence of substrate-use (Frey et al., 2013; Manzoni et al., 2012; Steinweg et al., 2008; Tucker et al., 2013). Patterns for thermodynamic efficiency and carbon-use efficiency were similar: on average, efficiencies of microbial communities in soils receiving glucose, trehalose or phenol were 0.06 – 0.15 units lower when incubated at 20 °C than at 5 °C (Fig. 1). These differences are similar to previous studies (Frey et al., 2013; Schindlbacher et al., 2015; Steinweg et al., 2008; Tucker et al., 2013). In soils with alanine addition, effects of temperature on efficiency were more pronounced and up to 0.53 units lower at 20 °C (Fig. 1). The mechanism behind this observation is elusive, but the following two aspects may have contributed: (i) alanine is an amino acid and a source of nitrogen, and (ii) potential bias inherent in the experimental approaches applied.

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

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

efficiencies decreased more strongly with increased temperature in samples receiving alanine in comparison to thermodynamic efficiencies. In the present study, thermodynamic efficiency assumes that ^{13}C recovered in the DOC pool represents only unused added substrate, i.e. residual substrate. Microbial excretion of recently assimilated ^{13}C into the DOC pool would result in an underestimation whereas adsorption of substrate would result in an overestimation of thermodynamic efficiency. We cannot evaluate if microbial excretion contributes significantly to the ^{13}C DOC pool, but it is unlikely that 50 % recovery as residual substrate at 5 °C after 24 h (Table 2) is mainly due to excretion. Also, we have some indications that adsorption is unlikely to be a factor introducing an experimental bias: The recovery was 83 and 92 % of the alanine C added to Compost and Unamended soils incubated at 5 °C, respectively and K_2SO_4 extraction should have recovered alanine C from the soil matrix. Future research should focus on method development to differentiate residual substrate from secreted ^{13}C -compounds, e.g. estimation of metabolites in soil solution using ^1H -NMR metabolomics (Hwang and Shaka, 1995; Tiziani et al., 2008). The methodological biases for thermodynamic efficiency mentioned above are also valid for carbon-use efficiency. In addition, assessment of microbial biomass via fumigation-extraction may be biased by non-uniform incorporation of substrate-C into microbial biochemical components in short-term incubation experiments (Marstorp and Witter, 1999; Witter and Dahlin, 1995). Results from substrate incorporation into biomass showed larger variation than measurements of residual substrate (Table 2). Therefore, it seems more likely that carbon-use efficiency values underestimate efficiency in samples receiving alanine due to a poor recovery of C incorporated into biomass.

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

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

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

4.2. Microbial community composition

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

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

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

Gram-positive bacteria were more active in utilising added substrates at 20 °C where efficiencies were low (Fig. 2) indicating a generally lower efficiency for this microbial group. The results should be treated with caution as temperature induced physiological alterations of PLFA profiles may introduce a bias. Gram-positive biomarkers are exclusively saturated fatty acids and these fatty acids tend to increase with temperature (Wixon and Balser, 2013). More abundant Gram-positive biomarkers may therefore be a result of changes in lipid composition of membranes as a direct response to temperature and not an increase in activity. Nevertheless, the higher activity at 20 °C cannot be explained exclusively by temperature induced alteration of lipid composition, because the higher activity of Gram-positive bacteria at 20 °C was also evident in the specific substrate incorporation (Fig. 2 E-H). The latter is less affected by changes in lipid composition, as it is the ratio of incorporated substrate per total abundance of a specific biomarker group. Changes in lipid composition should modify both values proportionally.

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

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

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

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

groups other than group A). At 5 °C, group A contributes with 50 % to the overall utilisation of a substrate and has an efficiency which is 0.20 (i.e. 20 %) higher than the efficiency of group B. If the overall substrate-use efficiency at the community level should decrease by 0.06 units with increasing temperature, group A would need to reduce its contribution to substrate utilisation to 20 %. This example illustrates that a large shift in active components of microbial communities combined with large differences in efficiencies between microbial groups are required to explain even the small changes in efficiency of 0.06 units. In our study, we observed efficiency differences up to 0.53 units, and consequently, shifts in the microbial community actively decomposing substrate are unlikely to cause the observed differences in substrate-use efficiency between the two temperatures. Therefore, changes in microbial physiology must be mainly responsible. In our experiment changes in microbial physiology were indicated by the different temperature sensitivities of microbial respiration and biomass production. Accumulation of CO₂ and heat production increased strongly while incorporation of substrate into biomass was less temperature sensitive, but with a tendency that substrate uptake decreased with increasing temperatures (Table 2). Similar differences in temperature sensitivity of these processes were also demonstrated by Schindlbacher et al. (2015) and discussed in a review by Manzoni et al. (2012). In summary, microbial respiration appears to be the main physiological driver for substrate-use efficiency, but small shifts in active microbial community may have contributed but to a minor extent in the present short-term incubation.

5. Conclusions

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

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