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Effect of temperature on metabolic activity of intact microbial communities: Evidence for altered metabolic pathway activity but not for increased maintenance respiration and reduced carbon use efficiency

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ABSTRACT

We used metabolic tracers and modeling to analyze the response of soil metabolism to a sudden change in temperature from 4 to 20 °C. We hypothesized that intact soil microbial communities would exhibit shifts in pentose phosphate pathway and glycolysis activity in the same way as is regularly observed for individual microorganisms in pure culture. We also hypothesized that increased maintenance respiration at higher temperature would result in greater energy production and reduced carbon use efficiency (CUE). Two hours after temperature increase, respiration increased almost 10-fold. Although all metabolic processes were increased, the relative activity of metabolic processes, biosynthesis, and energy production changed. Pentose phosphate pathway was reduced (17–20%), while activities of specific steps in glycolysis (51%) and Krebs cycle (7–13%) were increased. In contrast, only small but significant changes in biosynthesis (+2%), ATP production (−3%) and CUE (+2%) were observed. In a second experiment, we compared the metabolic responses to temperature increases in soils from high and low elevation. The shift in activity from pentose phosphate pathway to glycolysis with higher temperature was confirmed in both soils, but the responses of Krebs cycle, biosynthesis, ATP production, and CUE were site dependent. Our results indicate that 1) in response to temperature, communities behave biochemically similarly to single species and, 2) our understanding of temperature effects on CUE, energy production and use for maintenance and growth processes is still incomplete.

1. Introduction

Soil is the largest carbon (C) pool in the terrestrial biosphere (Houghton, 2007; Jobbágy and Jackson, 2000) and any change in its size may influence the atmospheric CO_2 concentration and feed back to ongoing climate change (Billings et al., 2010; Davidson and Janssens, 2006; Hungate et al., 2009; Schimel et al., 2000; Schlesinger and Andrews, 2000). Most soil microorganisms utilize organic C as a source of energy and biosynthetic precursors, while releasing CO_2 and contributing to long-term soil C storage. The regulation of energy (ATP, NADH, NADPH) production and consumption by intact soil microbial communities is not well-understood (Allison et al., 2010; Anderson and Domsch, 2010; Chapman and Gray, 1986; Del Giorgio and Cole, 1998; Schimel and Weintraub, 2003; Thiet et al., 2006) but fundamental to soil and therefore ecosystem C and N cycling (Burgin et al., 2011). Microbial energy status determines whether C is used for new microbial cells, ATP production, or CO_2 production, and subsequently affects whether nutrients are immobilized or mineralized.

A thorough understanding of the metabolic processes that underlie microbial energetics is an essential step toward the development of a deeper understanding of soil C and N cycling processes. Major questions associated with the response of soil C to climate change are related to microbial energetics: how do increased C inputs and higher temperatures change the balance between ATP production and consumption, and thereby CO_2 production, N mineralization, and soil organic matter formation (De Graaff et al., 2006; Hungate et al., 2009; Van Groenigen et al., 2006)? Microbial energy status is also related to soil organic matter breakdown and priming. One of the leading paradigms in soil organic matter turnover is that low energy yield prevents the microbial breakdown of recalcitrant compounds (Craine et al., 2007; Fontaine et al., 2007;

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Kuzyakov, 2010), perhaps explaining why soil organic matter turnover is stimulated by increased C input under elevated CO_2 (Carney et al., 2007; Langley et al., 2009; Taneva and Gonzalez-Meler, 2008). Litter quality also affects microbial energy status: anabolic and catabolic processes uncouple when high C:N ratio litter is utilized (Larsson et al., 1997; Manzoni and Porporato, 2009).

Carbon use efficiency (CUE, the proportion of substrate that is incorporated into microbial biomass) is an important microbial attribute, and included in most soil and ecosystem C cycling models. In general, the same value for CUE is applied to all ecosystems and environmental conditions (Manzoni and Porporato, 2009; Steinweg et al., 2008). Yet, changes in CUE have been observed. The C: N ratio of litter is negatively correlated with CUE (Manzoni et al., 2010). Additionally, CUE is affected by protozoa grazing (Frey et al., 2001), soil moisture content (Herron et al., 2009), glucose addition (Bremer and Kuikman, 1994; Shen and Bartha, 1996), and oxygen concentration (Parsons and Smith, 1989). From a theoretical perspective, and confirmed by some observations, higher temperatures increase maintenance energy demand and reduce CUE (Allison et al., 2010; Devêvre and Horwáth, 2000; Steinweg et al., 2008). Understanding the response of microbial metabolism to increased temperature is critical to predicting the future size of the terrestrial C sink (Bradford et al., 2008; Davidson and Janssens, 2006; Heimann and Reichstein, 2008).

Most of substrate taken up by microbial cells is processed through the central C metabolic pathways and most of the ATP and biosynthetic precursors that are needed for cell growth and maintenance are produced here (Almaas et al., 2004). These pathways include glycolysis, Krebs cycle, and pentose phosphate pathway. The rate of C flux through these various pathways varies with demand for ATP, NADH, NADPH and biosynthetic precursors, substrate availability, and environmental conditions (Gerstmeir et al., 2003; Rühl et al., 2010). For example, we have recently documented that when soil oxygen concentration is low, C flux through the Krebs cycle is reduced relative to glycolysis (Dijkstra et al., 2011). This shift is similar to what is known for individual microbial species (e.g., Chen et al., 2009; Hua and Shimizu, 1999).

Temperature decreases often cause a shift in activity from glycolysis and NADH production to pentose phosphate pathway and NADPH production in cultured microorganisms (Chung et al., 1976; Wittmann et al., 2007), and plant (Malone et al., 2006) and animal cells (Gallagher et al., 2009). This shift is thought to be in response to an increased NADPH demand needed to protect the cell against radical oxygen species (Ralser et al., 2007), increased pentose and NADPH demand for biosynthesis (Fuhrer and Sauer, 2009), or a way to maintain high Krebs cycle activity while circumventing temperature-inhibited steps in glycolysis (specifically phosphofructokinase is temperature sensitive; Gallagher et al., 2009; Malone et al., 2006).

We have conducted an experiment to examine the responses of fundamental biochemical processes in intact soil microbial communities to a change in temperature using the "parallel position-specific metabolic tracer labeling" technique (Dijkstra et al., 2011; in short the 'metabolic tracer method') in combination with 'metabolic modeling' (Dijkstra et al., in press) to determine C flux partitioning patterns across the reactions of the central metabolic network. In this method, position-specific ^{13}C-labeled metabolic tracers are added to soil in parallel incubations, taken up by microbial cells, and processed via ongoing metabolic processes. The amount added is so low that metabolic fluxes are not affected. The release of $^{13}CO_2$ from these metabolic tracers is measured and used to model the fluxes of C over the main biochemical pathways. From these fluxes, we estimate rates of biosynthesis, ATP production, and CUE. We hypothesize that in response to temperature increase, pentose phosphate pathway activity will decrease, while activity of glycolysis will be stimulated. We also hypothesize that ATP production will increase and CUE will decrease in response to higher temperatures.

2. Materials and methods

2.1. Experimental procedures

Soil (0–10 cm depth, A-horizon, Mollic Eutroboralf, $\delta^{13}C = -21.6‰$, C content = 1.5%, N content = 0.11%; pH 6) was collected in ponderosa pine forest from an area (approximately 100 m^2) covered with *Bouteloua gracilis* in an otherwise species-rich meadow (35.41541° N; longitude −111.6695° W, 2344 m above sea level) on 23 September 2010. Soil was sieved (2 mm mesh) and incubated at field capacity (27.2% gravimetric water content, determined according to Haubensak et al., 2002). Fifty g of soil was weighed into specimen cups that were preincubated in Mason jars ($n = 6$; 473 ml volume; Jarden Company, Rye, NY, USA) at 4 °C for 2 (replicates 1–3) and 4 days (replicates 4–6) at 5 °C.

The metabolic tracer technique consisted of adding small amounts of position-specific ^{13}C-labeled glucose and pyruvate (1-^{13}C pyruvate, 2,3-^{13}C pyruvate, 1-^{13}C glucose, and uniformly labeled (U-^{13}C) glucose) to soil in parallel incubations (Dijkstra et al., 2011). The metabolic tracers were added at low concentrations, to minimize their effect on ongoing metabolic processes, and were quickly taken up and utilized in microbial processes.

On the day of analysis, half of the jars were transported to the laboratory (20 °C), while the other half remained at 4 °C. After transfer, we waited 2 h until the temperature of the soil was within 2 °C of air temperature (20 °C). Subsequently, jars were opened and headspace was refreshed. After closing, 10 ml of pure CO_2 ($\delta^{13}C$ −6.8‰) was added to the headspace and concentration and isotope composition of CO_2 in each jar were determined after 30 min. The injection of pure CO_2 was carried out to satisfy the requirement of the Picarro G1101-*i* CO_2 cavity ring-down isotope spectrometer (Picarro Inc., Sunnyvale, California, USA) for a CO_2 concentration between 300 and 2000 μmol mol^{-1}. Subsequent dilution of the sample with CO_2-free air was done to create a volume large enough for 10 min sample analysis.

Each replicate consisted of five jars: control, 1-^{13}C labeled pyruvate, 2,3-^{13}C labeled pyruvate, 1-^{13}C glucose, and U-^{13}C glucose. After the first headspace sample was taken, two ml of a 3.6 mmol L^{-1} position-specific 1-^{13}C or 2,3-^{13}C labeled sodium pyruvate solution, or two ml of a 1.8 mmol L^{-1} 1-^{13}C or uniformly labeled (U-^{13}C) glucose solution was injected through a septum onto the surface of the soil. Each jar received 0.43 μmol tracer-C per g soil. Control soil received a similar amount of unlabeled glucose (first three replicates) or pyruvate (second three replicates). Pyruvate and glucose isotopologues were 99 atom% ^{13}C-enriched at the indicated C positions (Cambridge Isotope Laboratories, Andover, Massachusetts, USA).

Isotope composition and concentration of CO_2 were determined before and 10, 20, 40, 60, and 120 min after tracer addition at 20 °C, and before and after 30, 60, 120, 180 and 240 min at 4 °C. At each time, 10 ml of headspace air was collected, injected into a Tedlar air-sample bag (Zefon International, Ocala, Florida), and diluted with CO_2-free air. Jars were not opened between measurements. The production of $^{13}CO_2$ from metabolic tracers was calculated from the $^{13}CO_2$ produced from soil with metabolic tracer corrected for $^{13}CO_2$ produced from control soil. We determined the ratio of $^{13}CO_2$ produced from one isotopologue relative to that from the other isotopologue of the same metabolic tracer as follows:

$$C_1/C_{23}\,\text{ratio} = \frac{^{13}CO_2\,\text{production from}\,1-{}^{13}\text{C pyruvate}}{^{13}CO_2\,\text{production from}\,2,3-{}^{13}\text{C pyruvate}}. \quad (1)$$

Similarly,

$$C_U/C_1 \text{ ratio} = \frac{^{13}CO_2 \text{ production from U} - ^{13}C \text{ glucose}}{^{13}CO_2 \text{ production from 1} - ^{13}C \text{ glucose}}. \quad (2)$$

Any sorption of tracer to mineral and organic surfaces or incomplete uptake was assumed similar for both isotopologues, and therefore did not affect above ratios (Dijkstra et al., 2011).

Soil respiration rates were determined immediately following isotope measurements. Jars were opened and headspace atmosphere was replaced. Two hours later, an initial headspace sample was analyzed for CO_2 concentration with a LICOR 6262. This measurement was repeated over several days.

In a second experiment, we compared the metabolic responses to temperature change for two additional soil communities. The C. Hart Merriam Elevation Gradient consists of five sites along the San Francisco Peaks in Northern Arizona (Dijkstra et al., 2006; Blankinship et al., 2010). We chose the highest (2620 m above sea level; Pachic Udic Argiboroll; meadow in mixed conifer forest life zone) and the lowest site (1760 m; Typic Haplustand; cool desert grassland life zone) that were developed from volcanic parent material. We followed the same metabolic tracer procedure as described above for ponderosa pine forest soil ($n = 4$), except measurements of $^{13}CO_2$ were done 20 and 60 min after metabolic tracer addition.

2.2. Metabolic flux model

The model metabolic network for intact soil microbial communities is a simplification and generalization of existing models used for individual microbial species (e.g., Klapa et al., 2003; Morgan and Rhodes, 2002; Schmidt et al., 1997; Wiechert, 2001; Yang et al., 2005) and applied to soil communities (Fig. 1; Dijkstra et al., in press). This model simulates the fate of individual C atoms through glycolysis, Krebs cycle, pentose phosphate pathway, anaplerotic reactions (pyruvate carboxylase), and a variety of pathways that consume metabolites for the purpose of biosynthesis (Table 2). The model consists of a stoichiometric flux model (Fig. 1) in combination with atom-mapping-matrices and label identification vectors (Dijkstra et al., in press). The model is used to calculate how much and by which process C from different positions in metabolites is released as CO_2 thereby providing an estimate of the $^{13}CO_2$ production ratios for different metabolic tracers (eqs. (1) and (2)).

Several reactions and metabolite pools are lumped, for example, dihydroxyacetone-P, phosphoglycerate, and phosphoenolpyruvate are combined with glyceraldehyde-P. Fluxes (v_1–v_{21}) are net fluxes and are assumed greater or equal to zero (in the direction of the arrows). We assume steady state for metabolite pools; this assumption is not made for the much larger biomass pools. Carbon dioxide is produced by four decarboxylating reactions: 6-phosphogluconate dehydrogenase (reaction part of v_{10}), pyruvate dehydrogenase (v_5), isocitrate dehydrogenase (v_7), and α-ketoglutarate dehydrogenase (reaction part of v_8). The smaller contributions from other processes such as amino acid synthesis are ignored (Yang et al., 2005). Carbon dioxide used in anaplerosis is assumed unlabeled. We also assume that the microbial community uses glucose as their only C-substrate. We further assume a constant proportional precursor demand that is not affected by temperature. The proportional precursor demand is the relative requirement for metabolic precursors feeding biosynthesis reactions (v_{14}–v_{21}). The precursor requirement is a function of the chemical composition of microbial cells, estimated from published values for fungi, gram-positive and gram-negative bacteria using 1:1:1 = fungi: gram-positive bacteria: gram-negative bacteria as community composition (G6P:F1,6P:RU5P:GAP:PYR:ACCO:OAA:αKG = 1:0.32: 2.68:5.20:6.20:7.07:4.27:3.11) (Dijkstra et al., in press). Changes in proportional precursor demand and community composition have only small effects on flux calculations (Dijkstra et al., in press). The proportional precursor requirement does not dictate the rate of microbial growth in the model. The metabolic analysis is a community measurement, reflecting the sum of fluxes across all species and activities in soil.

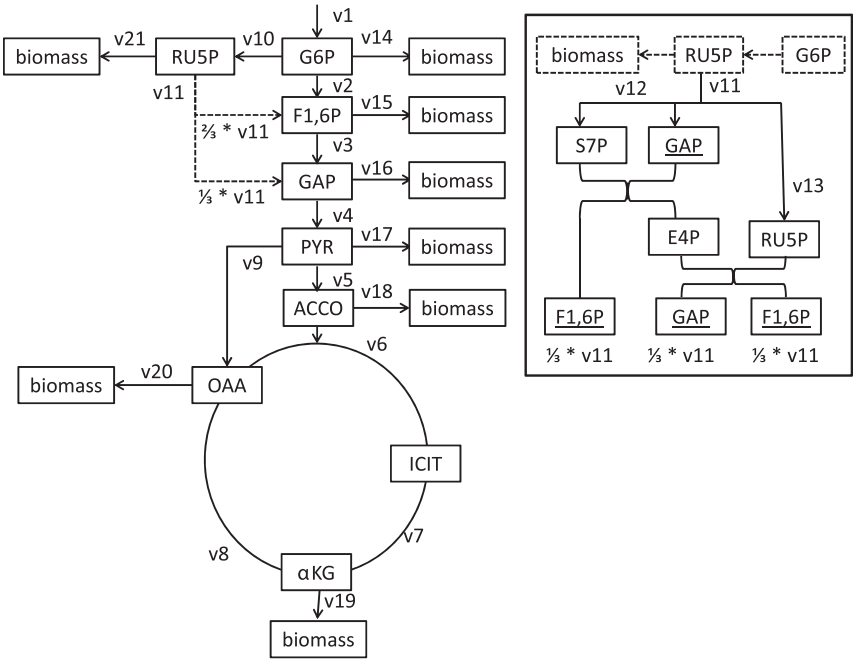

Fig. 1. A simple model for metabolic processes in soil microbial communities. Flux rates (v_2–v_{21}) are normalized relative to glucose uptake rate (v_1, set at 100) on a molar basis. Insert depicts details of the pentose phosphate pathway. Abbreviations: G6P, glucose-6P; F1,6P, fructose-1,6P_2; GAP, glyceraldehyde-P; PYR, pyruvate; ACCO, acetyl-CoA; ICIT, isocitrate; αKG, α-ketoglutarate; OAA, oxaloacetate; RU5P, ribulose-5P; S7P, sedoheptulose-7P; E4P, erythrose-4P (from Dijkstra et al., in press).

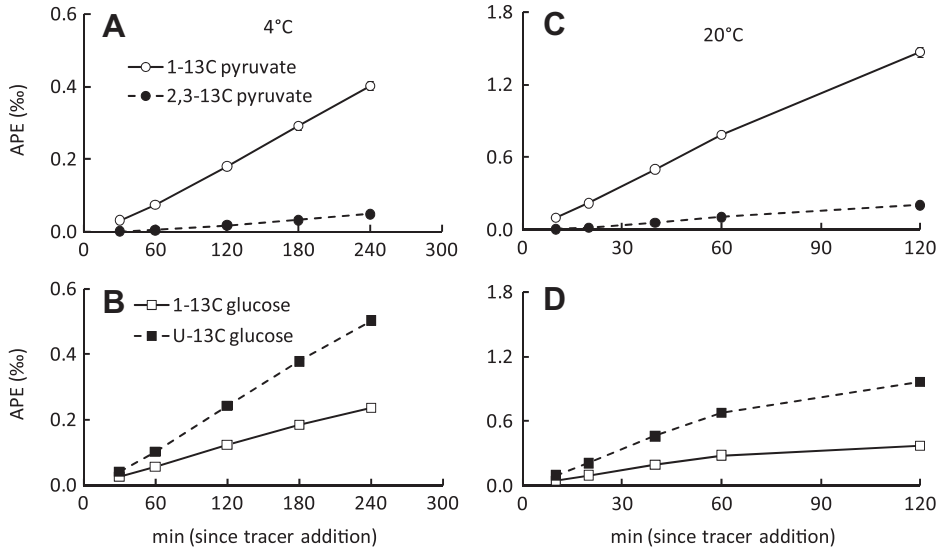

Fig. 2. Position-specific $^{13}CO_2$ production (expressed in atom percent excess, %) from pyruvate (A, C; circles) and glucose (B, D; squares) metabolic tracer isotopologues at 4 °C (A, B) and 2 h after transfer to 20 °C (C, D) in ponderosa pine forest soil ($n = 6$). Standard error ($n = 6$) is mostly smaller than the symbols.

The metabolic model consists of 21 reactions (including the reactions of the pentose phosphate pathway – Fig. 1). The reactions of the reductive branch of the pentose phosphate pathway are fully determined if v_{11} is known (Fig. 1 insert), reducing the model to 19 variables. Each node generates one equation assuming input equals output. This creates a set of 9 equations with 19 unknowns. To solve these equations, we need to estimate values for 10 unknown variables. By expressing all rates relative to v_1 (glucose uptake rate set at 100%), the number of unknowns is reduced to nine. Seven variables (v_{15}–v_{21}) are estimated by assuming constant proportional precursor demand, while the remaining two unknowns (v_{10} and v_{14}) are derived from experimentally determined metabolic tracer ratios (eqs. (1) and (2)).

The model equations are solved using 'Solver', a linear programming tool in Excel, by varying v_{10} and v_{14} reactions rates until $^{13}CO_2$ production ratios (eqs. (1) and (2)) calculated by the model match experimentally observed ratios, providing unique solutions for all rates under conditions that all fluxes are greater than or equal to zero in the direction of the arrows. The reaction rates and ATP production (normalized relative to v_1) are calculated by the model. Total energy production (ATP equivalents) was estimated using the modeled flux rates and the energy produced per reaction (Dijkstra et al., in press), assuming that NADPH and NADH produce 2.5 ATP and $FADH_2$ equals 1.5 ATP (Nelson and Cox, 2008). Finally, CUE is calculated as

$$\mathrm{CUE} = \frac{6 \times v_1 - \sum CO_2}{6 \times v_1}. \quad (3)$$

3. Results

The temperature of the soil was within 2 °C of room temperature 90 min after transfer. We conducted the tracer addition 30 min later. Soil respiration was almost 10 times higher at 20 °C than at 4 °C (1.05 μg CO_2–C g^{-1} soil h^{-1}; s.e. 0.03; $n = 6$ at high temperature, and 0.11 μg CO_2–C g^{-1} soil h^{-1}; s.e. 0.003; $n = 6$ at low temperature). This respiration rate was constant over 28 h (20 °C) and 122 h (4 °C). The $^{13}CO_2$ production from metabolic tracers was linear for 4 h at 4 °C, but only for 60 min at 20 °C (Fig. 2). The $^{13}CO_2$ production from glucose decreased after 60 min at 20 °C, an indication of metabolic tracer depletion. The decreased CO_2 production occurred concurrently for the two glucose isotopologues, confirming that isotopologues of a metabolic tracer behave similarly in soil and microbial cells. We determined the ratios of $^{13}CO_2$ production for each isotopologue pair (eqs. (1) and (2)) over the linear part of the curve (Fig. 2). The ratios of isotopologue-dependent $^{13}CO_2$ production were significantly affected by temperature: the C_1/C_{23} ratio for pyruvate decreased while the C_U/C_1 ratio for glucose increased in response to temperature increase (Table 1).

Model equations were solved separately for each replicate ($n = 6$). All reaction rates, expressed relative to glucose uptake rate, were significantly different from zero ($P < 0.05$; except v_1, which was set at 100% for both temperatures) and significantly affected by temperature (Table 2). The largest temperature effects were found in the first reactions of glycolysis and pentose phosphate pathway: a 17% reduction of C flowing into the pentose phosphate pathway (oxidative branch), a 20% reduction of C cycling back to glycolysis (non-oxidative branch), and a 51% increase in C flux between G6P and F1,6P (v_2; Fig. 3). Additionally, warming stimulated Krebs cycle activity and biosynthesis reactions relative to glucose uptake rate. We calculated energy production (ATP, NADH, NADPH, and $FADH_2$) rate relative to glucose uptake rate, and CUE based on model reaction rates (Table 3). Most energy was produced in the form of NADH, followed by NADPH. Transfer from low to high temperature increased ATP, NADH and $FADH_2$ production, but decreased NADPH production. ATP production per unit glucose consumed decreased slightly by 3%. It was assumed that isocitrate dehydrogenase (v_7) was NADP-dependent, although assuming this reaction produced NADH instead did not affect these conclusions.

We calculated the total substrate used (as moles glucose per g soil per h) from total CO_2 production divided by CUE (eq. (3)). As expected, total substrate consumption and ATP production per g soil were much higher at 20 °C than at 4 °C. However, C flux partitioning

Table 1
Effect of temperature on ratio of $^{13}CO_2$ production of metabolic tracer isotopologue pairs (±se, $n = 6$).

Metabolic tracer ratio	4 °C	20 °C	P
Pyruvate 1-^{13}C/2,3-^{13}C	7.82 (0.12)	6.97 (0.26)	<0.05
Glucose U-^{13}C/1-^{13}C	2.20 (0.04)	2.48 (0.02)	<0.05

Table 2
Reaction rates at 4 and 20 °C modeled from metabolic tracer experiments using pyruvate and glucose isotopologue pairs. For abbreviations see Fig. 1.

#	Reaction	4 °C	SE	20 °C	SE	*P*
v_1	Glucose → G6P	100	–	100	–	–
v_2	G6P → F1,6P	23.5	2.6	35.4	0.6	<0.01
v_3	F1,6P → 2 GAP	123.7	1.6	131.3	0.4	<0.01
v_4	GAP → PYR	120.0	0.6	123.1	0.2	<0.01
v_5	PYR → ACCO + CO_2	58.2	0.3	60.4	0.3	<0.01
v_6	ACCO + OAA → ICIT	26.0	0.15	27.7	0.3	<0.01
v_7	ICIT → αKG + CO_2	26.0	0.15	27.7	0.3	<0.01
v_8	αKG → OAA	11.9	0.14	13.4	0.38	<0.01
v_9	PYR + CO_2 → OAA	33.6	0.2	34.1	0.1	<0.05
v_{10}	G6P → RU5P + CO_2	72.0	2.6	60.0	0.6	<0.01
v_{11}	2 RU5P → S7P + GAP	59.8	2.7	47.6	0.7	<0.01
v_{12}	S7P + GAP → F1,6P + E4P	39.9	1.8	31.7	0.4	<0.01
v_{13}	RU5P + E4P → F1,6P + GAP	19.9	0.9	15.9	0.2	<0.01
v_{14}	G6P → biomass	4.55	0.02	4.62	0.01	<0.05
v_{15}	F1,6P → biomass	1.48	0.01	1.50	0.01	<0.05
v_{16}	GAP → biomass	23.6	0.12	24.0	0.07	<0.05
v_{17}	PYR → biomass	28.2	0.15	28.6	0.09	<0.05
v_{18}	ACCO → biomass	32.1	0.17	32.6	0.10	<0.05
v_{19}	αKG → biomass	14.1	0.07	14.4	0.04	<0.05
v_{20}	OAA → biomass	19.4	0.10	19.7	0.06	<0.05
v_{21}	RU5P → biomass	12.2	0.06	12.4	0.04	<0.05

over the different metabolic processes changed in response to temperature. We conclude that although large shifts in metabolic flux patterns were observed in response to temperature increase from 4 °C to 20 °C, these changes had only small consequences for biosynthesis and ATP production per g substrate.

Results from the second experiment were mostly in line with results described so far: large shifts in C flux from pentose phosphate pathway to glycolysis were observed after transfer to a higher temperature for both soils (interaction not significant; Table 4). However, the response to temperature increase of Krebs cycle activity (v_{6-8}), anaplerotic (v_9) and biomass reactions (v_{14}–v_{21}), and consequently CUE and ATP production differed significantly between soils. The low elevation soil exhibited only a small increase in Krebs cycle activity, a small decrease in ATP production, while CUE increased slightly (low temperature CUE = 0.72, s.e. = 0.008, $n = 6$; high temperature CUE = 0.73, s.e. = 0.009, $n = 4$). High elevation soil showed large increases in Krebs cycle activity, a small increase in energy production, while CUE was not significantly affected (low temperature CUE = 0.73; s.e. = 0.008, $n = 4$; high temperature CUE = 0.73, s.e. = 0.008, $n = 4$).

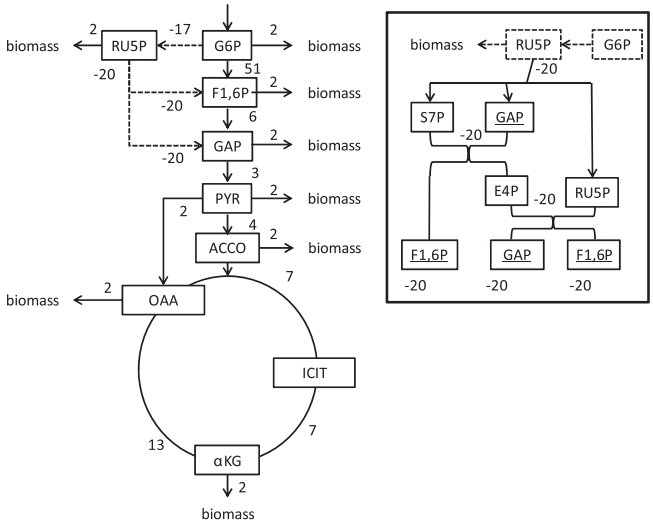

Fig. 3. Percentage change in reaction rates of metabolic processes in ponderosa pine forest soil in response to temperature increase from 4 °C to 20 °C. For absolute rates, see Table 2.

4. Discussion

4.1. Metabolic changes in soils associated with temperature shifts

We observed large and significant shifts in C flux from pentose phosphate pathway (v_{10}, v_{11}, v_{12}, v_{13}) to glycolysis (v_2) in response to a temperature increase from 4 to 20 °C for three soils along an elevation gradient. Krebs cycle activity was increased slightly for low elevation soils, but increased strongly in the high elevation soil. The ATP production, biosynthesis and CUE per unit substrate utilized were only slightly affected. ATP production increased by 1.5% for high elevation soil but declined in the other two soils in response to temperature increase. CUE followed the opposite pattern of ATP production, but changes were small (<2.5%).

The temperature change imposed on the microbial communities was very large (16 °C in 2 h), especially in the context of climate change, where small changes in average temperature are important. However, these large and fast changes in temperature are ecologically relevant. For example, high Andean soils experienced up to 40 °C increase during the day (Navas, 1997) with up to 20 °C increase per hour (Navas, 1996). Similarly, while air temperature increased by 10 °C at a high elevation site in Hawaii, soil surface temperature increased almost 20 °C (Juvik et al., 1993). At our high elevation site, a 20 °C change in soil surface temperature (5 cm depth) within 2 h is a normal occurrence when snow cover is absent.

The temperature shift altered metabolism in this short-term experiment. We can only speculate about longer-term temperature effects. Contin et al. (2000) observed increased ATP concentrations per g microbial biomass for communities incubated at higher temperatures, but these differences became evident only 13 days after temperature change. The slow response of ATP production may be associated with changes in community composition, C and N substrate availability, or physiological adaptation to growth temperature (Bradford et al., 2008; Knorr et al., 2005; Kruse et al., 2011; Zhang et al., 2005), and would therefore not be observed in this short-term experiment. We did find significantly lower Krebs cycle rates (8–17%), higher biosynthesis (2.3%) and CUE (2.0%), but lower ATP production (4%) for the high elevation soil compared to the low elevation soil when tested at 4 °C. When tested at 20 °C, soils from high and low elevation exhibited no significant metabolic differences.

4.2. Plasticity of pentose phosphate pathway and glycolysis

Warming shifted C flux from pentose phosphate pathway to glycolysis in these three soils (Fig. 3, Tables 2 and 4). Such redirection of C flux between pentose phosphate pathway and glycolysis in response to temperature has been observed for a broad range of organisms. For example, *Bacillus cereus* grown at 7 °C exhibited higher pentose phosphate pathway but lower glycolysis activity than when grown at 20 and 32 °C (Chung et al., 1976). Similarly, *Escherichia coli* lowered pentose phosphate pathway activity and increased glycolysis activity in response to a temperature increase (Luo et al., 2008; Weber et al., 2002; Wittmann et al., 2007). Potato tuber disks exhibited greater pentose phosphate pathway enzyme capacity and *in vivo* flux rates at lower temperatures (Malone et al., 2006; Wagner et al., 1987). On the other hand, the ratio of glycolysis to plastidic pentose phosphate pathway activity in soybean cotyledons increased at lower temperatures (Iyer et al., 2008). Finally, hypothermia in rats increased pentose

Table 3
Response of ATP, NADH, NADPH, and $FADH_2$ production and energy sum in ATP equivalents (moles per mole glucose), CUE, glucose uptake rate (nmol glucose g^{-1} soil h^{-1}), and total ATP production (nmol ATP g^{-1} soil h^{-1}) in ponderosa pine forest soil to a temperature increase from 4 to 20 °C (means and se; $n = 6$). Glucose uptake rate is calculated as respiration rate (μmol CO_2–C g^{-1} soil h^{-1})/(6 × CUE). Total energy produced in soil is the product of glucose uptake and total ATP produced mol^{-1} glucose.

	ATP	NADH	NADPH	$FADH_2$	Sum (ATP)	CUE	Glucose uptake	Total energy
4 °C	0.56 (0.003)	2.02 (0.01)	1.70 (0.05)	0.12 (0.001)	10.04 (0.11)	0.73 (0.004)	2.10 (0.01)	21.1 (0.3)
20 °C	0.60 (0.007)	2.10 (0.01)	1.48 (0.01)	0.13 (0.004)	9.75 (0.06)	0.75 (0.002)	19.5 (0.05)	190 (1.7)
P	<0.01	<0.01	<0.01	<0.01	<0.05	<0.05	<0.01	<0.01

phosphate pathway and decreased glycolysis activity (Gallagher et al., 2009; Kaibara et al., 1999).

A high pentose phosphate pathway activity may be related to increased pentose precursor demand, for example, for DNA or RNA synthesis, but may also signify an increased demand for NADPH (Fuhrer and Sauer, 2009). NADPH is used as an energy source for protein and lipid synthesis, and for protecting cells against oxidative stress (Nelson and Cox, 2008). Pentose phosphate pathway activity is generally increased at high oxygen concentrations or in the presence of reactive oxygen species, and decreased under low oxygen or anaerobic conditions (Chen et al., 2011; Fredlund et al., 2004; Godon et al., 1998; Gombert et al., 2001; Le Goffe et al., 2002; Ralser et al., 2007; Williams et al., 2008). Glucose-6P dehydrogenase (part of v_{10}) is controlled by the NADPH/NADP ratio and increases when NADPH consumption is high. Phosphofructokinase (enzyme catalyzing the rate-limiting step of aerobic glycolysis; part of v_2) may be especially sensitive to low temperatures (Malone et al., 2006), or inhibited because of changed energy status and metabolite concentrations (Gallagher et al., 2009; Tai et al., 2007; Wittmann et al., 2007). On the other hand, glucose-6P dehydrogenase (enzyme catalyzing the rate-limiting step of the pentose phosphate pathway – Gallagher et al., 2009; part of v_{10}) is stimulated by low NADPH/NADP ratios and high glucose-6P concentration (Kruse et al., 2011; Larsson et al., 1997; Moritz et al., 2000, but see Fuhrer and Sauer, 2009). An active pentose phosphate shunt at low temperature would enable high Krebs cycle activity, bypassing a temperature-inhibited step in glycolysis.

Table 4
Responses of two soils (high elevation mixed conifer soil and low elevation grassland desert soil) to a shift in temperature from 4 to 20 °C (%; $n = 4$), calculated as 100 × (warm–cold)/cold), and *P* value from ANOVA (temperature × site). Reaction rates, CUE, and ATP production were modeled from metabolic tracer experiments using pyruvate and glucose isotopologue pairs. Reaction numbers correspond to notation in Table 2. Significant values are underlined.

#	% Response to temperature increase		*P*-values		
	Low elevation	High elevation	Elevation	Temperature	Interaction
v_1	–	–	–	–	–
v_2	45.8	26.3	0.84	<0.01	0.15
v_3	6.1	4.1	0.14	<0.01	0.22
v_4	2.5	2.4	0.28	<0.01	0.99
v_5	2.7	5.7	0.11	<0.01	0.06
v_6	3.3	13.1	0.11	<0.01	<0.05
v_7	3.3	13.1	0.11	<0.01	<0.05
v_8	4.3	26.5	0.12	<0.01	<0.05
v_9	2.1	−0.9	0.31	0.45	<0.05
v_{10}	−18.3	−11.1	0.86	<0.01	0.15
v_{11}	−22.5	−13.4	0.90	<0.01	0.13
v_{12}	−22.5	−13.4	0.90	<0.01	0.13
v_{13}	−22.5	−13.4	0.90	<0.01	0.13
v_{14}	2.1	−0.9	0.31	0.45	<0.05
v_{15}	2.1	−0.9	0.31	0.45	<0.05
v_{16}	2.1	−0.9	0.31	0.45	<0.05
v_{17}	2.1	−0.9	0.31	0.45	<0.05
v_{18}	2.1	−0.9	0.31	0.45	<0.05
v_{19}	2.1	−0.9	0.31	0.45	<0.05
v_{20}	2.1	−0.9	0.31	0.45	<0.05
v_{21}	2.1	−0.9	0.31	0.45	<0.05
CUE	2.2	−0.5	0.37	0.22	<0.05
ATP production	−4.2	1.5	0.33	0.36	<0.05

4.3. Soil C cycling, temperature and microbial metabolism

In soil and ecosystem sciences, temperature responses of soil respiration are usually described using Arrhenius equations or by calculating a Q_{10} value of respiration (Davidson et al., 2006; Knorr et al., 2005; Manzoni and Porporato, 2009; Zhu and Cheng, 2011). Both equations assume an exponential increase of respiration with temperature; an assumption that is increasingly being challenged (Atkin et al., 2000; Davidson et al., 2006; Kruse et al., 2011). A more mechanistic approach widely applied to respiration involves the concept of energy cost for growth, maintenance, and ion uptake processes (Amthor, 2000; Anderson and Domsch, 2010; Blagodatsky and Richter, 1998; Chapman and Gray, 1986; Van Bodegom, 2007). This relatively simple concept connects substrate utilization to organismal physiology and growth. Maintenance respiration (energy costs associated with protein turnover, membrane repair, ion gradient upkeep, motility) is expected to increase with rising temperature. Consequently, less C is available for growth and CUE declines at higher temperatures (Allison et al., 2010; Steinweg et al., 2008). However, neither approach provides mechanistic insights into the various processes involved and their relationships with temperature (Anderson and Domsch, 2010; Davidson et al., 2006; Van Bodegom, 2007).

Our results show that short-term warming causes only small changes in ATP production, biosynthesis and CUE (relative to glucose uptake rates), sometimes changing in a direction opposite to expectations. The absence of a consistent increase in ATP production and decrease in biosynthesis and CUE with rising temperature suggests that the proportion of maintenance respiration in total respiration was unchanged. However, the large shift from NADPH to NADH production with higher temperature suggests that nature of energy-consuming processes underlying maintenance changed dramatically. We interpret this as an indication that maintenance processes themselves changed, although the total amount of energy required did not. These processes shifted from protection against oxidative damage (or other NADPH consumption) to NADH-consuming processes (for example maintenance of proton gradients and to a small degree increased biosynthesis). We conclude, with others (e.g. Davidson et al., 2006; Van Bodegom, 2007) that physiologically and ecologically complex mechanisms lie underneath the response of microbial respiration to temperature. We tentatively conclude that concepts of maintenance and growth respiration do not capture the complex reality of changes in microbial biochemistry and physiology, and should be used carefully.

The estimates of CUE are at the high end of the range of values obtained for soils (e.g., Frey et al., 2001) and microorganisms in pure culture (e.g., Roels, 1980). At this moment, not enough experience exists with metabolic flux modeling in soils to conclude definitively that these CUE estimates are representative for soils, or alternatively

that limitations in modeling and measuring of metabolic processes are the cause for these high values. However, the following arguments need to be considered. 1) In past studies, CUE was often determined after several days, introducing uncertainty whether primary processing of substrates were measured, or whether secondary processing through higher trophic levels or remobilization of internal reserves influenced the estimate of this variable. Our methodology provides an almost instantaneous estimate of CUE over 2 h (Fig. 2). 2) Pure-culture experiments are often conducted with high substrate availability. These concentrations are much higher than realistic for soils. Excess substrate availability can induce glucose-repression of Krebs cycle activity (Gombert et al., 2001; Blencke et al., 2003, see also Manzoni and Porporato, 2009 on N-inhibition and C-overflow hypotheses). This glucose repression will negatively affect energy production and CUE. 3) We used disturbed soil, thereby stimulating soil activity. This likely reduces the proportion of energy needed for maintenance processes relative to that for growth and nutrient uptake. This will increase CUE.

4.4. Modeling of metabolic processes in soil

Very little is known about metabolic processes in soil. We have taken, hesitantly, the first few steps on the road toward fundamental biochemical analysis of soil C processes. There is no doubt that many obstacles need to be overcome. The following discussion identifies two of these obstacles.

4.4.1. Relationship between microbial community composition, metabolic capacities, and soil activity

Genomic studies show that the diversity of soil microbial communities is astonishingly high (Fierer et al., 2009), incorporating an amazing metabolic diversity. The soil microbial community contains autotrophic and heterotrophic organisms, species with complete Krebs cycles, non-cyclic Krebs 'cycles', and species with alternative configurations of glycolysis or pentose phosphate pathways. For example, *Acidobacteria* can make up more than 50% of the microbial community (Fierer et al., 2009; Jones et al., 2009). Although we know very little about this recently discovered group of organisms, some cultured isolates are lacking fumarase, and likely have non-cyclic Krebs 'cycles' (KEGG, http://www.genome.jp/kegg-bin/show_pathway?map01100). Modeling of soil metabolic processes needs to accomodate this metabolic variability. This can be done by combining information on community composition with estimates of relative activity. For example, *Acidobacteria* are likely very slow-growing organisms (Janssen, 2006; Ward et al., 2009) and, although abundant, probably make up only a small percentage of total soil activity. Other species with alternative metabolic systems are obligate anaerobes, and may similarly be of low importance in overall activity of aerobic soils. At this stage, we assume that the metabolic activity in soils is dominated by aerobic organisms with full Krebs cycle, glycolysis and pentose phosphate pathways. Future work will need to identify whether this simplification is warranted, and how contributions of less active components of the microbial community can be incorporated into the community metabolic analysis.

4.4.2. Substrates for microbial processes

Surface soils experience a high input of substrate in the form of plant litter and root materials, while soil disturbance further enhances C availability (Hart et al., 1994). These substrates are dominated by carbohydrates, especially glucose, a ubiquitous molecule present in cellulose, hemicellulose, starch, and soluble form. However, under other circumstances, for example in rhizosphere or for microbes breaking down lignin, waxes or lipids, substrate may include organic acids, such as oxalate, citrate and acetate (Dennis et al., 2010; Plassard and Fransson, 2009). Utilization of organic acids instead of glucose induces gluconeogenesis: the production of carbohydrates from small organic C compounds by a process that is the reverse of glycolysis (Nelson and Cox, 2008). We showed previously (Dijkstra et al., 2011) that in the presence of succinate, a gluconeogenic substrate, $^{13}CO_2$ production from pyruvate was strongly repressed. This would be even stronger for the glucose tracer. Future research needs to develop approaches to identify when substrates other than carbohydrates are utilized and correctly model their biochemical consequences.

We believe that a combination of genomic, metabolomic, and stable isotope techniques can help to unravel the biochemistry of intact soil communities. The fact that the biochemistry of intact soil microbial communities and individual species responded similarly to temperature changes (this study), gluconeogenic substrates and anaerobic conditions (Dijkstra et al., 2011) is encouraging in this respect.

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