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High carbon use efficiency in soil microbial communities is related to balanced growth, not storage compound synthesis

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ABSTRACT

The efficiency with which microbes use substrate (Carbon Use Efficiency or CUE) to make new microbial biomass is an important variable in soil and ecosystem C cycling models. It is generally assumed that CUE of microbial activity in soils is low, however measured values vary widely. It is hypothesized that high values of CUE observed in especially short-term incubations reflect the build-up of storage compounds in response to a sudden increase in substrate availability and are therefore not representative of CUE of microbial activity in unamended soil.

To test this hypothesis, we measured the $^{13}CO_2$ release from six position-specific ^{13}C-labeled glucose isotopomers in ponderosa pine and piñon-juniper soil. We compared this position-specific CO_2 production pattern with patterns expected for 1) balanced microbial growth (synthesis of all compounds needed to build new microbial cells) at a low, medium, or high CUE, and 2) synthesis of storage compounds (glycogen, tri-palmitoyl-glycerol, and polyhydroxybutyrate).

Results of this study show that synthesis of storage compounds is not responsible for the observed high CUE. Instead, it is the position-specific CO_2 production expected for balanced growth and high CUE that best matches the observed CO_2 production pattern in these two soils. Comparison with published studies suggests that the amount of glucose added in this study is too low and the duration of the experiment too short to affect microbial metabolism. We conclude that the hypothesis of high CUE in undisturbed soil microbial communities remains viable and worthy of further testing.

1. Introduction

Heterotrophic microbes use organic carbon (C) compounds to synthesize cellular compounds while releasing some substrate-C as CO_2. Which compounds are synthesized depends on the physiology of the cells (active growth and division, survival when substrate availability is low, dormancy). It is currently not possible to determine directly the compounds that are produced. It seems plausible that the microbial community consists of cells in all possible physiological states at any time, unless there are synchronizing events, such as a simultaneous depletion of substrate in all soil niches or a sudden increase in substrate availability. The C Use Efficiency (CUE; biomass-C synthesized per substrate-C consumed; mol C/mol C) of the soil microbial community is an important ecosystem variable that influences what proportion of organic C utilized is released to the atmosphere as CO_2 or potentially remains in the soil as organic matter in living cells or dead soil organic matter (Billings and Ballantyne, 2013; Bradford, 2013; Hagerty et al., 2014). Indirectly, CUE also determines whether nutrients such as nitrogen (N) or phosphate are immobilized or mineralized (Manzoni et al., 2012; Sinsabaugh et al., 2013). Consequently, an improved understanding of CUE is important for soil C and N cycling models (Allison et al., 2010; Manzoni et al., 2012; Wieder et al., 2013; Hagerty et al., 2014; Li et al., 2014). The CUE is a

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function of the cellular demand for energy and biosynthesis, and therefore a function of the physiological state and the type of compounds that are being produced. When only energy is required (such as for cell maintenance), CUE is close or equal to zero (Chapman and Gray, 1986; Amthor, 2000).

Because of low C availability in soil and the supposedly recalcitrant nature of soil organic matter, the CUE of the microbial community is often assumed to be low (Anderson and Domsch, 2010; Manzoni et al., 2012; Sinsabaugh et al., 2013; Reischke et al., 2015). The limited substrate available is used to satisfy energy demands for cell maintenance with little left for growth. However, many studies find high values of CUE (0.6 and higher; e.g., Brant et al., 2006; Dijkstra et al., 2011a,b; Frey et al., 2013; van Groenigen et al., 2013; Hagerty et al., 2014; Steinweg et al., 2008; Thiet et al., 2006; Tucker et al., 2013; Ziegler et al., 2005). The average CUE observed in soil is 0.55 (Manzoni et al., 2012; Sinsabaugh et al., 2013). This value is remarkably close to the average maximum value of CUE observed in pure culture studies (~0.6; Blagodatskaya et al., 2014; Roels, 1980; Sinsabaugh et al., 2013), but below the theoretical thermodynamic maximal CUE of growth on glucose (0.88–1.0; Gommers et al., 1988; Heijnen, 2010; Heijnen and van Dijken, 1992; Manzoni et al., 2012; Roels, 1980; Xiao and van Briesen, 2006). The average CUE for soil is much higher than that found in aquatic ecosystems (~0.3; Hobbie and Hobbie, 2013; Manzoni et al., 2012; Sinsabaugh et al., 2013). This large discrepancy in CUE raised concerns (Hobbie and Hobbie, 2013; Sinsabaugh et al., 2013), prompting a critical evaluation of methods used to determine community CUE (Sinsabaugh et al., 2013).

The measurement of CUE often involves adding (^{13}C-enriched) substrates. It is suggested that high substrate additions alter CUE, either increasing (Sinsabaugh et al., 2013; van Groenigen et al., 2013) or decreasing it (van Groenigen et al., 2013; Russell, 2007). Specifically for short-term experiments, it is hypothesized that high CUE values may not represent microbial balanced growth (that is, the synthesis of all compounds needed to build new cells), but instead may be the result of rapid uptake of substrate followed by synthesis of storage compounds (Nguyen and Guckert, 2001; Hill et al., 2008; Sinsabaugh et al., 2013; Blagodatskaya et al., 2014; Reischke et al., 2014, 2015). Although this still represents an increase in biomass, for a sound understanding of C cycling in soil ecosystems, it is important to distinguish between CUE during long-term microbial activity and that where microbes temporarily allocate C to storage synthesis associated with a sudden and temporary increase in substrate availability (Sinsabaugh et al., 2013). Microbial cells can store substrate as starch, glycogen, trehalose, extracellular polysaccharides (Wilson et al., 2010), polyhydroxyalkanoates and storage lipids (Olsson and Johansen, 2000; Lu et al., 2009). However, measurements of storage synthesis in soil have not been made.

In this study, we evaluate four mutually exclusive hypotheses: 1) the microbial community uses substrate for maintenance only (CUE = 0); 2) the microbial community exhibits balanced growth but an overall low CUE (CUE = 0.3 as suggested by Sinsabaugh et al., 2013), 3) the microbial community exhibits a high CUE but "unbalanced" growth where biosynthesis is limited to storage compound production (glycogen, lipids, or polyhydroxybutyrate), and 4) the microbial community exhibits balanced growth at high CUE (0.6; close to the maximal CUE in pure culture studies).

We conducted an incubation experiment with six position-specific ^{13}C-labeled glucose isotopomers and two soils from northern Arizona, USA. We compared the observed pattern of position-specific CO_2 production with patterns predicted for balanced microbial growth at varying CUE (CUE = 0, 0.3, or 0.6) and storage synthesis (glycogen, tri-palmitoyl-glycerol – TPG – and polyhydroxybutyrate - PHB). By comparing our experimental methods and results with published studies of responses of microbial growth to substrate addition, we tested a fifth hypothesis that the increase in substrate availability changed the CUE of the microbial community. We show that the observed position-specific CO_2 production resembles patterns expected for balanced growth at high CUE, and does not match CO_2 production patterns of any combination of low or medium CUE and storage compound synthesis. According to currently published research results, these results were not affected by the change in substrate availability.

2. Materials and methods

2.1. Experimental procedures

We collected soil (0–10 cm depth) from two locations along the C. Hart Merriam Elevation Gradient (www.nau.edu/Ecoss/) near Flagstaff, Arizona in the fall of 2012. The highest site (2340 m elevation, mean annual temperature (MAT) 8 °C, mean annual precipitation (MAP) 660 mm) was a small open area in a ponderosa pine (*Pinus ponderosa*) stand covered with blue grama (*Bouteloua gracilis*) grass. Soil was a Mollic Eutroboralf (C content 1.5%, N content 0.11%; Dijkstra et al., 2006). The second site (2020 m elevation, MAT 10 °C, MAP 380 mm) was an intercanopy space in a piñon-juniper stand (*Pinus edulis*, *Juniperus monosperma*) also covered with blue grama grass. Soil type was a Calcic Haplustand (C content 1.7%, N content 0.16%; Dijkstra et al., 2006). Soil was sieved (2 mm mesh) and stored at 4 °C until used.

We weighed 40 g of sieved soil into a specimen cup and placed it in a Mason jar (473 ml) equipped with an airtight lid and septum (n = 4). Soil moisture content was adjusted to field capacity (0.272 and 0.300 g water g^{-1} soil dry weight for respectively ponderosa pine and piñon-juniper soil) and soil was incubated overnight in the dark at room temperature (21 °C). The next morning, jars were opened, headspace atmosphere was replaced with lab air, and, after closing the jar, 10 ml of pure CO_2 was added to the headspace. This addition of pure CO_2 was needed to have enough CO_2 in 10 ml headspace gas samples for the Picarro 2101-*i* CO_2 isotope spectrometer (Picarro Inc, Sunnyvale, CA) to measure isotope ratios within the calibrated range of concentrations (Dijkstra et al., 2011a). After 30 min and before glucose isotopologue addition, a 10 ml headspace gas sample was taken (time zero).

We used glucose (^{13}C-labeled in C_1, C_2, C_3, C_4, C_5, C_6 and uniformly (U) labeled) as the metabolic tracer (99 atom fraction %; Cambridge Isotope Laboratories, Andover, Massachusetts). Two ml of a 1.79 mM glucose isotopomer solution was added to each specimen cup (0.536 μmol glucose-C g^{-1} soil; n = 4). Because of the large number of isotopologues, replicates, each consisting of seven glucose isotopologue incubations, were done on successive days. Ten ml headspace gas samples were taken 20, 40, and 60 min after tracer addition and analyzed for isotope composition with the Picarro CO_2 isotope analyzer. The isotope composition of headspace CO_2 was expressed as atom fraction excess (%; Coplen, 2011) and plotted against time. We determined the slope of atom fraction excess (calculated as the difference between the atom fraction at t = 1 and the atom fraction at t = 0) for the period that the CO_2 production rate was constant (40 min, Fig. 3A) and calculated the ratio of position-specific CO_2 production rates as follows:

$$\frac{C_x}{C_U} = \frac{^{13}CO_2 \text{ production from } x - {}^{13}C \text{ glucose}}{^{13}CO_2 \text{ production from } U - {}^{13}C \text{ glucose}} \quad (1)$$

where x stands for each of the six C-atoms in glucose and U for the uniformly labeled glucose isotopologue. Ratios were calculated for each replicate.

2.2. Modeling

We used the metabolic model of the central C metabolic network (CCMN) as described in Dijkstra et al. (2011a) with small modifications (Fig. 1). In short, this model included glycolysis, pentose phosphate pathway (PP-pathway), TCA cycle, pyruvate carboxylase as the anaplerotic reaction, and eight precursor-consuming biosynthesis reactions. The model assumed that glucose is the only substrate for cell metabolism. The model included consumption of CCMN intermediates for biomass synthesis. The model did not make any assumptions about microbial growth rates or CUE, but assumed that all biomass synthesis reactions consumed precursors in constant proportion (in other words a constant chemical composition of the cell). The proportional precursor demand (demand for precursors to enable balanced growth) was br1:br2:br3:br4:br5:br6:br7:br8 = 1:0.32:9.29:11.63:13.20:5.01:7.44:4.89 representative of Gram-negative bacteria, estimated from pure culture studies (Dijkstra et al., 2011a). We compared the position-specific CO_2 fluxes for Gram-negative bacteria with CO_2 fluxes for Gram-positive bacteria and fungi which have slightly different precursor demands and chemical composition (see below; Dijkstra et al., 2011a). The model calculated the fate of each C atom in glucose and the probability that it is released as CO_2 by pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase or phosphogluconate dehydrogenase. Carbon use efficiency of the microbial community was calculated from the model as

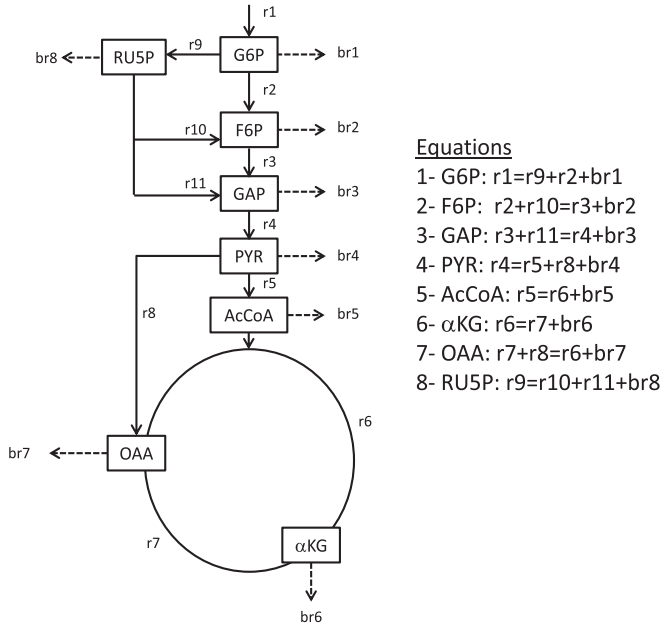

Fig. 1. Model and mass balance equations for calculation of fluxes through the central C metabolic network (after Dijkstra et al., 2011a). Relative to a previous version, pentose phosphate pathway and TCA cycle representations are simplified by combining several reactions. These alterations do not change model outcomes. Flux rates (reactions r2–r11 and biosynthesis reactions br1–br8) are normalized to glucose uptake rate (r1, set at 100) on a molar basis. Abbreviations: G6P, glucose-6P; F1,6P, fructose-1,6P2; GAP, glyceraldehyde-P; PYR, pyruvate; ACCO, acetyl-CoA; ICIT, isocitrate; αKG, α-ketoglutarate; OAA, oxaloacetate; RU5P, ribulose-5P; S7P, sedoheptulose-7P; E4P, erythrose-4P.

$$CUE = 1 - \frac{\sum(r5,\ r6,\ r7,\ r9)}{6 \times r1}. \quad (2)$$

with *r5*, *r6*, *r7*, *r9* as the rate of the CO_2-releasing reactions r5, r6, r7, and r9 respectively, and *r1* as the rate of substrate uptake r1 (Fig. 1). For further details, see Dijkstra et al. (2011a,b), van Groenigen et al. (2013), and supplemental information with Hagerty et al. (2014).

Molar flux rates of the CCMN processes (relative to glucose uptake) were estimated by matching observed and modeled patterns of position-specific CO_2 production. In the previous version of this model, isotopologue pairs of glucose and pyruvate were used. In this study, we solved the model using the six isotopologue ratios (eq. (1)) of glucose. A model solution was calculated by minimizing the sum of squares (SS) of the difference between observed and predicted ratios for the six glucose isotopomers by altering the reaction rates *r9* and *br1* (Fig. 1) using the Excel linear programming tool Solver. A local minimum of SS was sometimes observed when model calculations were initiated with *r9* and *br1* equal to zero. For these solutions, R^2 was low and the regression had a negative slope. These results were avoided when initiating the Solver procedure with r9 and br1 greater than 0.5.

2.3. Calculation of position-specific CO_2 production for balanced growth with low, medium, and high CUE

The metabolic model (Fig. 1) is typically used to find the flux rates through the CCMN processes and CUE by matching modeled glucose (and pyruvate) isotopomer ratios to ratios observed in soil. However, the model can also be used in reverse to calculate glucose position-specific CO_2 production rates (or isotopomer ratios) for hypothetical situations. We used the model to calculate the hypothetical position-specific CO_2 production rates for CUE equal to 0 (*i.e.*, only synthesis of ATP for maintenance processes), CUE equal to 0.3 (*i.e.*, most likely CUE in soil ecosystems as proposed by Sinsabaugh et al., 2013), and CUE equal to 0.6 (*i.e.*, CUE similar to the maximum observed in pure culture studies). For CUE equal to 0.3 or 0.6, position-specific CO_2 production was calculated assuming balanced microbial growth (synthesis of all compounds to build new microbial biomass plus energy for maintenance). Because the position-specific CO_2 production patterns were strongly influenced by the activity of the PP-pathway, we modeled CO_2 production patterns for minimal and maximal PP-pathway activity for Gram-negative bacteria separately (see above for proportional precursor demand). For maximal PP-pathway activity, we set the value of *r2* (Fig. 1) to zero, so that all flux was directed via the PP-pathway. Substrate was returned to the glycolysis as fructose-6P and glyceraldehyde-3P. For minimal PP-pathway activity, the flux of substrate into the PP-pathway was set to that required for growth (*r9* = *br8*), but no substrate was cycled through the PP-pathway back into glycolysis. For these two situations, *br1* was then manually changed until the calculated value of CUE equaled the desired value (0, 0.3, or 0.6).

To evaluate the sensitivity of position-specific CO_2 production rates for variation in proportional precursor demand, we compared the position-specific CO_2 production for the situation of balanced growth, CUE equal to 0.6, and minimal and maximal activity of the PP-pathway for Gram-negative bacteria (see above), Gram-positive bacteria (1:0.47:5.01:5.53:6.28:3.27:4.28:2.54) and fungi (1:0.18:1.30:1.45:1.72:1.04:1.08:0.60).

2.4. Calculation of position-specific CO_2 production for storage compound synthesis

We also calculated the CUE and position-specific CO_2 production of cells that synthesize glycogen (as an example of carbohydrate

storage), PHB (an example of polyhydroxyalkanoates), and TPG (an example of lipid storage) by taking into account the amount of ATP and precursors needed for biosynthesis. Information on synthesis pathway stoichiometry and energy demand was obtained from MetaCyc.org (Caspi et al., 2014) and is detailed in sections 2.4.1–2.4.3.

We manually calculated the position-specific CO_2 production rates for synthesis of glycogen, TPG, and PHB, again for minimal (all C flows via glycolysis) and maximal activity of the PP-pathway (all C flows via the PP-pathway). Glycogen synthesis included glucose uptake, phosphorylation, and polymerization into glycogen with ATP as the energy donor. ATP was provided by the complete oxidation of a small fraction of glucose to CO_2. Tri-palmitoyl-glycerol was synthesized with acetyl-CoA as the precursor for the fatty acids and dihydroxyacetone-P as the precursor for the glycerol backbone. The ATP needed for this process was produced during the formation of acetyl-CoA via glycolysis and PP-pathway. The extra energy for desaturation of fatty acids came from complete oxidation of a small amount of glucose when PP-pathway activity was minimal. When PP-pathway activity was maximal, enough ATP was produced for fatty acid production and subsequent desaturation reactions. Synthesis of PHB was accomplished with acetyl-CoA as the only precursor, and all required energy was produced during acetyl-CoA production via glycolysis or PP-pathway.

During the synthesis of acetyl-CoA from glucose with minimal PP-pathway activity, C_3 and C_4 of glucose were released as CO_2 by pyruvate dehydrogenase. The remainder (acetyl-CoA) was used for TPG and PHB synthesis (Fig. 2). In this case, C_3 and C_4 of the original glucose molecule were released as CO_2, while C_1, C_2, C_5 and C_6 of glucose were incorporated in acetyl-CoA and ended up in fatty acids or polyhydroxybutyrate.

The breakdown of glucose to acetyl-CoA via the PP-pathway was more complex as C_1 of glucose was lost in the first few reactions, and the C_2 and C_3 were rearranged to form fructose-6P and glyceraldehyde-3P (Fig. 2). As part of the PP-pathway, glucose was decarboxylated and the pentose sugars were rearranged to fructose-6P and glyceraldehyde-3P in a ratio of 2:1. Fructose-6P then broke into two molecules glyceraldehyde-3P, which mixed with glyceraldehyde-3P retrieved from the PP-pathway. The next step was the release of CO_2 by pyruvate dehydrogenase to form acetyl-CoA.

For energy production, we assumed that 1 NAD(P)H was equivalent to 2.5 ATP, 1 $FADH_2$ produced 1.5 ATP, and 1 GTP was equal to 1 ATP and were readily exchangeable.

2.4.1. Glycogen

We used glycogen as an example of a storage compound derived from glucose-6P precursors (Wilson et al., 2010). ATP cost for making glycogen included glucose uptake (1 ATP – de Kok et al., 2012), phosphorylation (1 ATP), and transformation to UDP-glucose and polymerization (cost for regenerating UTP is 1 ATP). For high glucose concentrations, glucose can enter microbial cells through facilitated transport, but in this analysis, we assumed low glucose concentrations where proton-coupled symport was more likely (Wilson-O'Brien et al., 2010). Glucose-6P precursor and energy demand for glycogen synthesis was described with the following reaction equation:

$$\text{glucose} + (\text{glucose})_n + 3\ \text{ATP} = (\text{glucose})_{n+1} + 3\ \text{ADP} + 3\ P_i.$$

The ATP needed for uptake and polymerization of 1 mol glucose into glycogen was produced from oxidation of 0.0968 mol glucose.

2.4.2. Tri-palmitoyl-glycerol

Storage lipids in fungi and bacteria include triacylglycerides (Alvarez and Steinbuchel, 2002; Kosa and Ragauskas, 2011). Palmitic acid is a common fatty acid in storage lipids in *Glomus* species (Olsson and Johansen, 2000). To make 1 mol TPG, 3 mol palmitic acid and 1 mol glycerol were consumed. To produce 3 mol palmitic acid, twelve mol glucose were metabolized to 24 mol acetyl-CoA while releasing 24 mol CO_2. An additional 0.5 mol glucose was needed to synthesize dihydroxyacetone-P, which was turned into 1 mol glycerol-P and combined with 3 mol palmitic acid to form 1 mol TPG. The stoichiometric reaction equation for the synthesis of TPG via glycolysis (minimal PP-pathway activity) was:

$$12.5\ \text{glucose} + 6\ \text{NAD(P)}^+ + 6\ H^+ + 16.5\ \text{ATP} = 24\ CO_2 + 6\ \text{NAD(P)H} + 16.5\ \text{ADP} + 16.5\ P_i + 1\ \text{TPG}.$$

Assuming that ATP and NAD(P)H were fully interchangeable, this reaction produced 0.5 mol ATP per mol TPG. The introduction of 1.5 double bonds per TPG (Olsson and Johansen, 2000) consumed

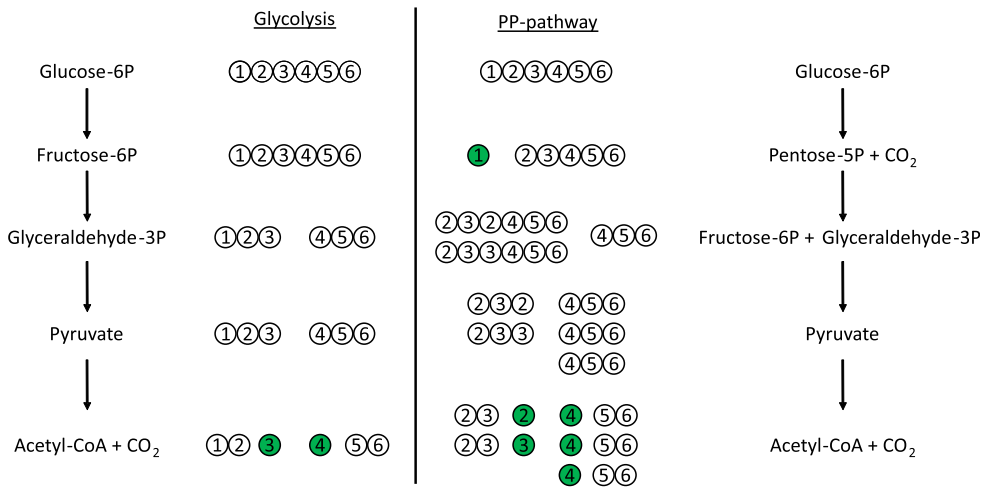

Fig. 2. Diagram of glucose breakdown via glycolysis (left) and PP-pathway (right) to acetyl-CoA and CO_2 (red circles). Number in circles refer to the C-atom position in the original glucose molecule. Filled circles are C atoms released as CO_2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

1 mol ATP per mol storage lipids. This required an additional 0.032 mol glucose, which was completely oxidized, producing ~0.19 mol CO_2. When substrate was directed via the PP-pathway (maximal PP-pathway activity), the stoichiometric reaction equation for TPG synthesis was:

$$15\ \text{glucose} + 36\ \text{NAD(P)}^+ + 6\ \text{H}^+ + 16.5\ \text{ATP} = 39\ \text{CO}_2 + 36\ \text{NAD(P)H} + 16.5\ \text{ADP} + 16.5\ \text{P}_i + 1\ \text{TPG}.$$

In this case, sufficient ATP was produced to drive desaturation reactions.

2.4.3. Polyhydroxybutyrate

Many bacterial species are able to synthesize polyhydroxyalkanoates as C and energy storage (Lu et al., 2009). Polyhydroxybutyrate is a representative of this class of compounds. For the synthesis of PHB, glucose was taken up by the cell, phosphorylated, and metabolized to acetyl-CoA. Two acetyl-CoA molecules were then combined into 3-hydroxy-butanoyl-CoA and polymerized to PHB, consuming 1 NADPH. The stoichiometric reaction equation for PHB synthesis via glycolysis (minimal PP-pathway activity) was:

$$1\ \text{glucose} + (\text{PHB})_n + 1\ \text{ADP} + 1\ \text{P}_i + 3\ \text{NAD(P)}^+ + 3\ \text{H}^+ = (\text{PHB})_{n+1} + 2\ \text{CO}_2 + 1\ \text{ATP} + 3\ \text{NAD(P)H}.$$

When glucose was directed into the PP-pathway (maximal PP-pathway activity), the equation for PHB synthesis was:

$$6\ \text{glucose} + (\text{PHB})_n + 4\ \text{ADP} + 4\ \text{P}_i + 27\ \text{NAD(P)}^+ + 27\ \text{H}^+ = (\text{PHB})_{n+5} + 16\ \text{CO}_2 + 4\ \text{ATP} + 27\ \text{NAD(P)H}.$$

All ATP required for these reactions was produced during the production of acetyl-CoA.

2.5. Statistical analysis

One-way analysis of variance was used to evaluate differences between soils. To determine which biochemical scenario (balanced growth with CUE = 0, CUE = 0.3, or CUE = 0.6, synthesis of storage compounds) best explained observed CO_2 production data, we assessed whether the 95% confidence interval of observed isotopomer ratios overlapped with model predictions. Correspondence between observed and predicted position-specific CO_2 production was evaluated using R^2, and closeness of slope and intercept to the expected 1:1 line.

3. Results

3.1. Observed position-specific CO_2 production

We measured the position-specific CO_2 production for glucose in ponderosa pine and piñon-juniper soil. The rate of CO_2 production from glucose isotopomers was constant for 40 min (Fig. 3A, results piñon-juniper soil not shown), after which it started to decline as was seen in previous studies (Dijkstra et al., 2011b). There were clear and significant differences in CO_2 production from different C atoms ($P < 0.05$): CO_2 production from C_1 was significantly higher than C_4, which was higher than C_2 and C_3, while C_5 and C_6 were the lowest (Fig. 3B). This pattern was the same for both soils. The CUE derived from modeling (following Dijkstra et al., 2011a with slight modifications) was not significantly different between the two soils (0.62 for ponderosa pine soil, 0.61 for piñon-juniper soil).

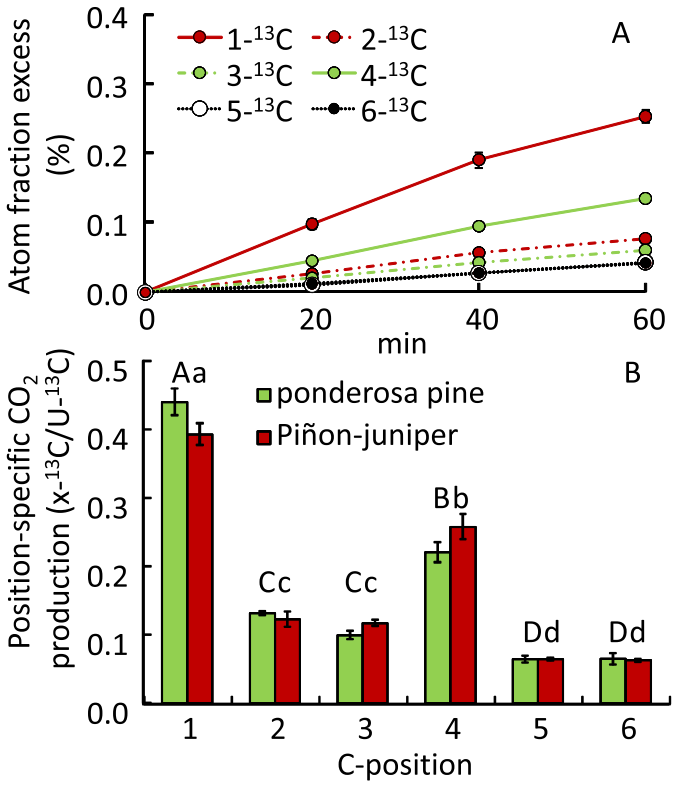

Fig. 3. Rates of CO_2 production for six glucose isotopomers in ponderosa pine soil (A; means and S.E.) and position-specific CO_2 production (relative to U–^{13}C labeled glucose; B; means and 95% confidence interval) for ponderosa pine and piñon-juniper soil. Letters indicate significant differences between C positions for ponderosa pine (upper case) and piñon-juniper (lower case) soil.

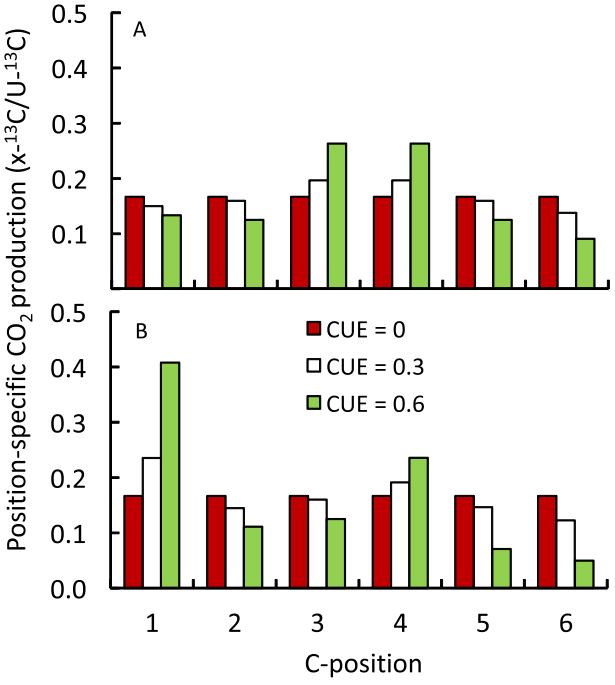

Fig. 4. Modeled position-specific CO_2 production (relative to U–^{13}C labeled glucose) for CUE = 0, 0.3, and 0.6 for minimal (A) and maximal (B) pentose phosphate pathway activity.

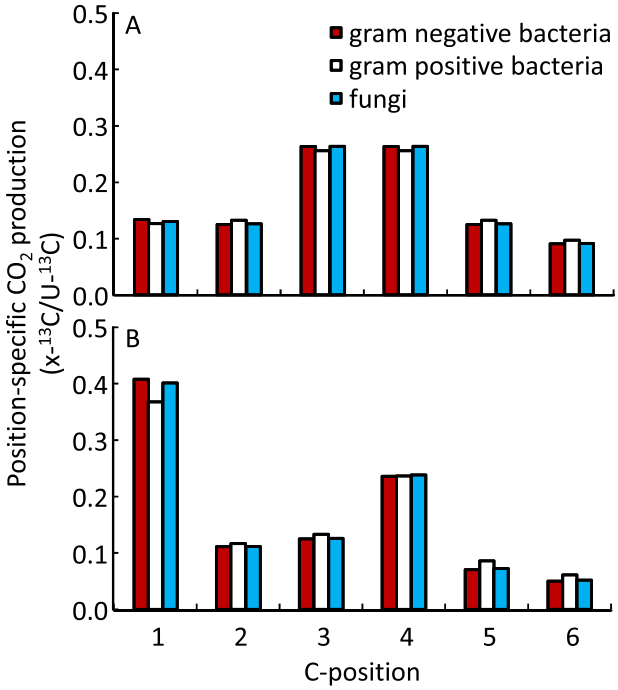

Fig. 5. Modeled position-specific CO_2 production (relative to U–^{13}C labeled glucose) for CUE = 0.6 for minimal (A) and maximal (B) pentose phosphate pathway activity for proportional precursor demand characteristic for Gram-negative bacteria, Gram-positive bacteria, and fungi.

Table 1
Carbon Use Efficiency of glycogen, tri-palmitoyl-glycerol, and polyhydroxybutyrate synthesis assuming minimal and maximal pentose phosphate pathway activity calculated from stoichiometry of synthesis reactions (eqs. (3)–(6)).

PP-pathway	Compound synthesized	CUE
Minimal	Glycogen	0.90
	Tri-palmitoyl-glycerol	0.68
	Polyhydroxybutyrate	0.67
Maximal	Glycogen	0.90
	Tri-palmitoyl-glycerol	0.57
	Polyhydroxybutyrate	0.56

3.2. Modeled position-specific CO_2 production

3.2.1. Balanced growth and varying CUE

Carbon use efficiency and activity of the PP-pathway had a large influence on the position-specific CO_2 production (Fig. 4). With maximal PP-pathway activity and high CUE, most of the CO_2 was produced from C_1 and C_4; in contrast, when PP-pathway activity was minimal, most CO_2 was produced from C_3 and C_4. With decreasing CUE, these differences became less pronounced. When CUE equaled 0, substrate was used only for synthesis of ATP and NAD(P)H, and all C positions were released as CO_2 at the same rate (ratio of C_x:C_U = 1:6).

The compounds produced to make bacterial or fungal cells differed slightly resulting in small differences in proportional precursor demand between Gram-negative and Gram-positive bacteria and fungi (Dijkstra et al., 2011a). These differences in proportional precursor demand had only a small effect on metabolic flux patterns and CUE (Dijkstra et al., 2011a,b; van Groenigen et al., 2013). This was also true for the position-specific CO_2 production (Fig. 5). For this reason, the results presented for Gram-negative bacteria were considered representative for any combination of fungi, Gram-positive and Gram-negative bacteria.

3.2.2. Storage compound synthesis

The CUE of storage compound synthesis was high (Table 1) although dependent on the form of storage (glycogen > TPG ≈ PHB). For TPG and PHB, the CUE was reduced when PP-pathway activity was high.

Complete oxidation of glucose to CO_2 was needed to provide the ATP for glycogen synthesis. This resulted in a position-specific CO_2 production that was equal for all C atoms (Table 2), similar to the situation with only maintenance energy (Fig. 4). The ATP required for TPG synthesis was produced during the breakdown of glucose to acetyl-CoA. Therefore, with minimal PP-pathway activity and all C flowing via glycolysis, CO_2 was only released during the pyruvate dehydrogenase reaction (Fig. 2 (left); C_3 and C_4 lost as CO_2; all other C atoms are incorporated into the fatty acids; Table 2). However, desaturation of palmitic acid (Olsson and Johansen, 2000) required an additional mol ATP per mol TPG (0.032 mol glucose, producing ~0.19 mol CO_2 evenly from all six C atoms, and reducing CUE from 0.680 to 0.678). This had only a minor effect on the position-specific CO_2 production. The breakdown of glucose to acetyl-CoA via the PP-pathway had a higher energy yield and most CO_2 was released from C_1 and C_4 (Fig. 2; Tables 1 and 2). The position-specific CO_2 production associated with PHB synthesis resembled that of TPG (Table 2).

We did not model the synthesis of other storage compounds (starch, extracellular polysaccharides, trehalose, other fatty acids, other polyhydroxyalkanoates). The metabolic pathways for these compounds were closely related to those for glycogen, TPG or PHB, and would likely result in similar CO_2 production patterns and CUE. The approach developed here can be used for the synthesis of other hypothetical reserve compounds, at least as long as the details of the biosynthetic pathways are known.

3.3. Correlation between measured and modeled position-specific CO_2 production

The correlation between measured and modeled position-specific CO_2 production was low for CUE = 0, glycogen synthesis, and all cases where PP-pathway activity was low (Table 3).

Table 2
Predicted CO_2 production patterns associated with glycogen, tri-palmitoyl-glycerol and polyhydroxybutyrate synthesis assuming minimal and maximal pentose phosphate pathway activity.

PP-pathway	Compound synthesized	C_1	C_2	C_3	C_4	C_5	C_6
Minimal	Glycogen	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$
	Tri-palmitoyl-glycerol	0.000$^{\$A}$	0.000$^{\$A}$	0.500$^{\$A}$	0.500$^{\$A}$	0.000$^{\$A}$	0.000$^{\$A}$
	Polyhydroxybutyrate	0.000$^{\$A}$	0.000$^{\$A}$	0.500$^{\$A}$	0.500$^{\$A}$	0.000$^{\$A}$	0.000$^{\$A}$
Maximal	Glycogen	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$	0.167$^{\$A}$
	Tri-palmitoyl-glycerol	0.385	0.128	0.128$^{\$}$	0.359$^{\$A}$	0.000$^{\$A}$	0.000$^{\$A}$
	Polyhydroxybutyrate	0.375$^{\$}$	0.125$^{\$A}$	0.125$^{\$}$	0.375$^{\$A}$	0.000$^{\$A}$	0.000$^{\$A}$

$^{\$}$ and ^{A} indicate significant differences with observed position-specific CO_2 production for ponderosa pine and piñon-juniper soil respectively.

Table 3
Correlation of observed position-specific CO_2 production pattern for ponderosa pine (first number) and piñon-juniper soil (second number) with modeled CO_2 production patterns for balanced growth (CUE = 0, CUE = 0.3. CUE = 0.6), and glycogen, tri-palmitoyl-glycerol, and polyhydroxybutyrate synthesis with minimal or maximal pentose phosphate pathway activity.

PP-pathway	CUE or compounds synthesized	R^2	Slope	Intercept
Minimal	CUE = 0	0.00/0.00	0.00/0.00	0.167/0.167
	CUE = 0.3	0.00/0.01	−0.01/0.02	0.168/0.164
	CUE = 0.6	0.00/0.04	0.03/0.12	0.162/0.146
	Glycogen	0.00/0.00	0.00/0.00	0.167/0.167
	Tri-palmitoyl-glycerol	0.00/0.01	−0.10/0.21	0.183/0.131
	Polyhydroxybutyrate	0.00/0.01	−0.10/0.21	0.183/0.131
Maximal	CUE = 0	0.00/0.00	0.00/0.00	0.167/0.167
	CUE = 0.3	0.91/0.95	0.27/0.30	0.121/0.115
	CUE = 0.6	0.98/0.99	0.92/1.03	0.010/−0.007
	Glycogen	0.00/0.00	0.00/0.00	0.167/0.167
	Tri-palmitoyl-glycerol	0.78/0.90	1.04/1.23	−0.010/−0.042
	Polyhydroxybutyrate	0.75/0.88	1.02/1.23	−0.007/−0.041

However, the modeled patterns of position-specific CO_2 production for maximal PP-pathway activity explained between 75 and 99% of the variance in the observed data. Although the modeled patterns of CUE = 0.3 and TPG and PHB synthesis (with maximal PP-pathway activity) exhibited high correlation coefficients (Table 3, Fig. 6), we found the best fit between observed and modeled position-specific CO_2 production for the hypothetical situation of balanced growth with a CUE = 0.6 and high PP-pathway activity (98–99% of variance explained; slope and intercept very close to the expected 1: 1 line).

Combining low CUE (CUE = 0 or 0.3) with storage synthesis decreased the correspondence between modeled and observed position-specific CO_2 production. This is easiest understood by focusing on one C position, for example C_1. Assume CUE = 0 combined with sudden synthesis of TPG. In that case, CO_2 from C_1 during TPG synthesis assuming maximal PP-pathway activity (0.385) is combined with CO_2 production from C_1 for CUE = 0 (0.167). This will reduce the CO_2 production from C_1, and increase the difference between predicted and observed CO_2 production for this C atom (0.44 or 0.43 for ponderosa pine and piñon-juniper soil respectively). In fact, any combination of medium or low CUE and storage synthesis resulted in CO_2 production patterns that deviated more from observed patterns than those associated with storage compound synthesis alone.

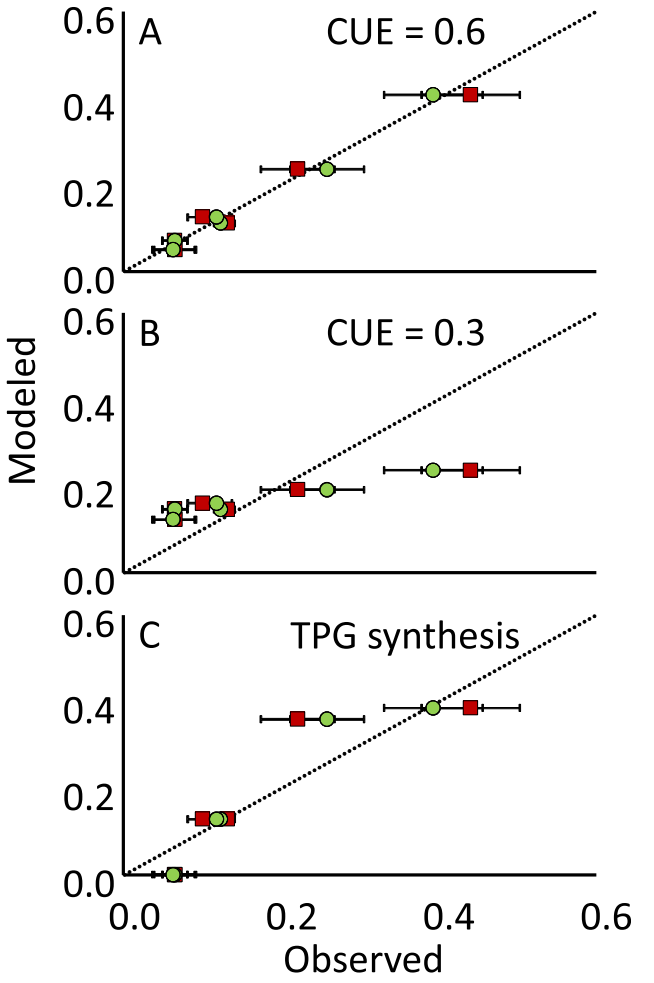

Fig. 6. Modeled versus observed position-specific CO_2 production (relative to U–^{13}C labeled glucose; means and 95% confidence interval) for ponderosa pine (red squares) and piñon-juniper soil (green circles) for modeled balanced growth with CUE = 0.6 (A), CUE = 0.3 (B), and tri-palmitoyl glycerol synthesis (C) with maximal pentose phosphate pathway activity. Dashed lines are the expected 1:1 relationships. Information on regression statistics is available in Table 3 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

4. Discussion

Determining the position-specific CO_2 production from ^{13}C-labeled compounds is a straightforward and quick way to test biochemically explicit hypotheses for microbial processes, including storage compound synthesis, in microbial communities. In this study, we tested the mutually exclusive hypotheses that CUE of microbial substrate use is zero (substrate used for maintenance only – Hypothesis 1), CUE is low (important role for maintenance energy demand – Hypothesis 2), CUE is high because of "unbalanced" growth (storage compound production – Hypothesis 3), or CUE is high associated with balanced growth (Hypothesis 4). Based on the evidence presented, we conclude that the soil microbial community had a high CUE associated with balanced growth (Hypothesis 4).

The CUE observed is in the same range as found on average for soil ecosystems (~0.55) and higher than that in aquatic ecosystems (~0.3; Manzoni et al., 2012; Sinsabaugh et al., 2013). The two soils in this study exhibit similar patterns, suggesting that the metabolic processes in these soils are similar. Studies using a broad range of soils are required to determine whether this is a general pattern in soils. The results from this study demonstrate that observations of high CUE in earlier studies (e.g., Nguyen and Guckert, 2001; Brant et al., 2006; Thiet et al., 2006; Hill et al., 2008; Steinweg et al., 2008; Dijkstra et al., 2011a,b; Frey et al., 2013; Tucker et al., 2013; van Groenigen et al., 2013; Hagerty et al., 2014) do not necessarily represent storage compound synthesis as sometimes suggested (Nguyen and Guckert, 2001; Hill et al., 2008; Sinsabaugh et al., 2013; Blagodatskaya et al., 2014; Reischke et al., 2014, 2015), but may be related to balanced microbial growth.

4.1. High CUE: a consequence of glucose addition?

Although the results from this study exclude storage compound synthesis as an artefact, it does not eliminate other possible artefacts. The high CUE and balanced growth observed in this experiment may not be representative of microbial activity in unamended soil but a response to the glucose addition used to measure CUE (Hypothesis 5). Several studies have suggested that glucose additions may alter microbial growth and CUE, either increasing

(Sinsabaugh et al., 2013; van Groenigen et al., 2013) or decreasing it (van Groenigen et al., 2013; Russell, 2002). In the following, we will discuss the influence of substrate addition on microbial growth and metabolism, specifically the effect of substrate concentration and response time.

Substrate additions used to determine CUE range from 0.8 nmol glucose-C g^{-1} soil (Nguyen and Guckert, 2001) to 61.5 μmol glucose-C g^{-1} soil (Thiet et al., 2006). The glucose applied in this experiment is at the low end of this range (0.536 μmol glucose-C g^{-1} soil), and well within the range of concentrations found in unamended soils (~1 nmol glucose-C g^{-1} soil – Fischer et al., 2007 - to 18 μmol g^{-1} soil – Jones and Darrah, 1995). Yet, high CUE is found in this and other short-term experiments with even lower glucose additions (0.8 nmol glucose-C g^{-1} soil, Nguyen and Guckert, 2001; 1.6 nmol glucose-C g^{-1} soil, Hill et al., 2008). We conclude that there is no evidence to suggest that CUE is high because of unnaturally high concentrations of substrate.

Anderson and Domsch (2010) and Reischke et al. (2014, 2015) find that glucose addition stimulates microbial growth, but only after a lag-phase of 8–14 h and at high glucose concentrations (>4.6–90 μmol glucose-C g^{-1} soil depending on soil type – Anderson and Domsch, 2010; >41.5 μmol glucose-C g^{-1} soil – Reischke et al., 2014; >16 μmol glucose-C g^{-1} soil – Reischke et al., 2015). These concentrations are higher than used in this experiment (0.536 μmol glucose-C g^{-1} soil), suggesting that the glucose additions used in this experiment, and those by Nguyen and Guckert (2001) and Hill et al. (2008), are too low and the incubation duration too short to induce microbial growth.

Furthermore, almost immediately after glucose addition, respiration increases, while microbial growth rates remain unaffected (Reischke et al., 2014, 2015). These observations imply that CUE decreases during the lag-phase in response to (a large) glucose addition. Similar declines in CUE are found in pure culture studies where glucose addition rates exceed maximum growth rates or when other nutrients than C limit growth (Russell and Cook 1995; Russell, 2007). However, the CUE measured in this experiment and by Nguyen and Guckert (2001) and Hill et al. (2008) are high, suggesting again that substrate additions used did not affect microbial metabolism.

We conclude, based on existing studies on soil microbial community growth that the glucose addition in this experiment is too low and incubation duration too short to induce microbial growth. Furthermore, CUE was high and not low as expected during a lag-phase after glucose addition. Finally, storage compound synthesis was ruled out as an artefact based on the observed position-specific CO_2 production patterns. Therefore, we tentatively conclude that the high CUE and balanced growth we observed is representative of CUE in unamended soil.

4.2. High CUE and maintenance energy requirements

The high CUE observed seems in contradiction to the idea that soil is a C-limited environment where most microbes are not growing or only grow slowly, and where maintenance energy demand dominates substrate use (Blagodatskaya and Kuzyakov, 2013; Reischke et al., 2015). Evidence of actively dividing microbes is found by Rousk et al. (2011) and Reischke et al. (2014, 2015) in soil without glucose additions, and in $^{18}O–H_2O$ stable isotope probing experiments (Schwartz, 2007). Blagodatskaya and Kuzyakov (2013) conclude from extensive literature review that about 0.1–2% of the soil microbial cells are actively growing and reproducing. This direct evidence of microbial growth in unamended soils indicates that at least a portion of the microbial community has a high CUE and balanced growth. Moreover, a low growth rate by itself, expected in C- and nutrient-limited soils, is not necessarily associated with a low CUE. For example, a 10-fold reduction in growth rate (0.388 h^{-1} to 0.044 h^{-1}) in *E. coli* pure cultures caused only a moderate reduction of CUE from 0.60 to 0.51 (Kayser et al., 2005). Likewise, Lin et al. (2009) find no change in CUE for *Geobacillus* growth rates ranging between 0.053 h^{-1} and 0.00078 h^{-1}.

A high CUE for the entire soil community is only possible if growing microbes with high CUE dominate microbial activity compared to microbes with low CUE. The community in soil is thought to consist of actively growing and dividing (0.1–2%), potentially growing (10–40%), and dormant microbes (remaining fraction; Blagodatskaya and Kuzyakov, 2013). To what degree the high CUE in a small, actively growing and dividing community is "diluted" by maintenance respiration of the inactive portion of the community may be calculated as follows. For simplicity, we assume that the active microbial fraction grows with a CUE near the highest values observed (CUE = 0.7), while the potentially active and dormant fractions conduct maintenance only (CUE = 0). Price and Sowers (2004) estimate ratios of metabolic rates of optimal-growth: maintenance: survival (dormancy) as 1: 10^{-3}: 10^{-6}. Applying these values to a community with 0.1–2% actively growing and dividing cells means that about 90–98% of substrate consumed is associated with actively growing microbes, and only 10–2% with potentially active or dormant microbes. A CUE of 0.7 for the actively growing community would then translate to a total community CUE of 0.64 (0.1% of community actively growing) or 0.69 (2% active). A similar argument is presented in Frey et al. (2001).

5. Conclusions

It is a well-established practice to use uniformly-labeled compounds to study microbial processes, including CUE. We show here that additional information is obtained by using position-specific ^{13}C-labeled compounds. This information can be used to test biochemically explicit hypotheses related to microbial physiology and biochemistry in soil microbial communities. We conclude that CUE in two soil microbial communities is high. This high CUE is not related to storage synthesis but to balanced growth, and appears to be unaffected by the small amount of glucose added.

The conclusion that the soil microbial community operates with a high CUE in soil environments has important and potential far-reaching consequences. It affects how we model microbial activity in soils and think about the relative importance of maintenance processes. As a result, microbial death (caused by viruses, grazing and predation) becomes more important as a key process in stabilizing microbial population size and community composition (Hagerty et al., 2014), suggesting a possible top-down control of microbial production by organisms at higher trophic levels. It also invites a rethinking of the recalcitrant nature of soil organic matter and its suitability as a microbial substrate, and, as a consequence, a rebalancing of the role of chemical vs physical protection in soil organic matter stabilization (von Lützow et al., 2006). We conclude that the hypothesis of high CUE in undisturbed soil remains viable and worthy of further testing.

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