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RESEARCH ARTICLE

Heavy and wet: The consequences of violating assumptions of measuring soil microbial growth efficiency using the ^{18}O water method

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Soils store more carbon than the biosphere and atmosphere combined, and the efficiency to which soil microorganisms allocate carbon to growth rather than respiration is increasingly considered a proxy for the soil capacity to store carbon. This carbon use efficiency (CUE) is measured via different methods, and more recently, the ^{18}O-H_2O method has been embraced as a significant improvement for measuring CUE of soil microbial communities. Based on extrapolating ^{18}O incorporation into DNA to new biomass, this measurement makes various implicit assumptions about the microbial community at hand. Here we conducted a literature review to evaluate how viable these assumptions are and then developed a mathematical model to test how violating them affects estimates of the growth component of CUE in soil. We applied this model to previously collected data from two kinds of soil microbial communities. By changing one parameter at a time, we confirmed our previous observation that CUE was reduced by fungal removal. Our results also show that depending on the microbial community composition, there can be substantial discrepancies between estimated and true microbial growth. Of the numerous implicit assumptions that might be violated, not accounting for the contribution of sources of oxygen other than extracellular water to DNA leads to a consistent underestimation of CUE. We present a framework that allows researchers to evaluate how their experimental conditions may influence their ^{18}O-H_2O-based CUE measurements and suggest the parameters that need further constraining to more accurately quantify growth and CUE.

Keywords: Microbial growth, Carbon use efficiency, Stable isotopes

Introduction

Soil microbes serve as key modulators of the global carbon cycle, transforming the massive amounts of carbon found in soil into biomass and waste products such as carbon dioxide. This partitioning of carbon into growth as opposed to respiration—or carbon use efficiency (CUE)—is increasingly measured in soils as a sensitive integrator of microbial response to the impacts of human activity such as elevated temperature (Frey et al., 2013), altered soil moisture (Herron et al., 2009; Zheng et al., 2019), land use change (Malik et al., 2018; Bölscher et al., 2020), and nutrient availability (Spohn et al., 2016). A range of carbon cycle models have shown that the degree to which soil organic matter is lost in a warmer world is contingent upon assumptions made about the value and environmental sensitivity of CUE (Allison et al., 2010; Frey et al., 2013; Li et al., 2014; Sistla et al., 2014; Wieder et al., 2015; Pold et al., 2019). Thus, measurements of CUE must be accurate for these models to validly predict changes in the soil carbon cycle in a changing world.

For many years, the study of CUE was restricted to examining the CUE of one substrate type at a time. In this method, a single isotopically labeled carbon source is added to the soil. Heavy carbon is partitioned by the cell into respiration and biomass, and CUE can be calculated as the ratio of heavy carbon collected from biomass to the sum collected from both biomass and CO_2 respiration. However, theoretical work suggests that this method overestimates "true" efficiency by measuring the uptake of simple labile compounds and not their integration into biomass (Hagerty et al., 2018; Geyer et al., 2019). ^{13}C methods may also overestimate CUE if the target compound preferentially enters anabolic pathways while nonlabeled substrates are used to generate energy (Gommers et al., 1988; Lehmeier et al., 2015). Furthermore, ^{13}C methods measure substrate use efficiency on a specific compound and do not capture differences in the efficiency of microbial growth on the wide repertoire of substrates available in the soil.

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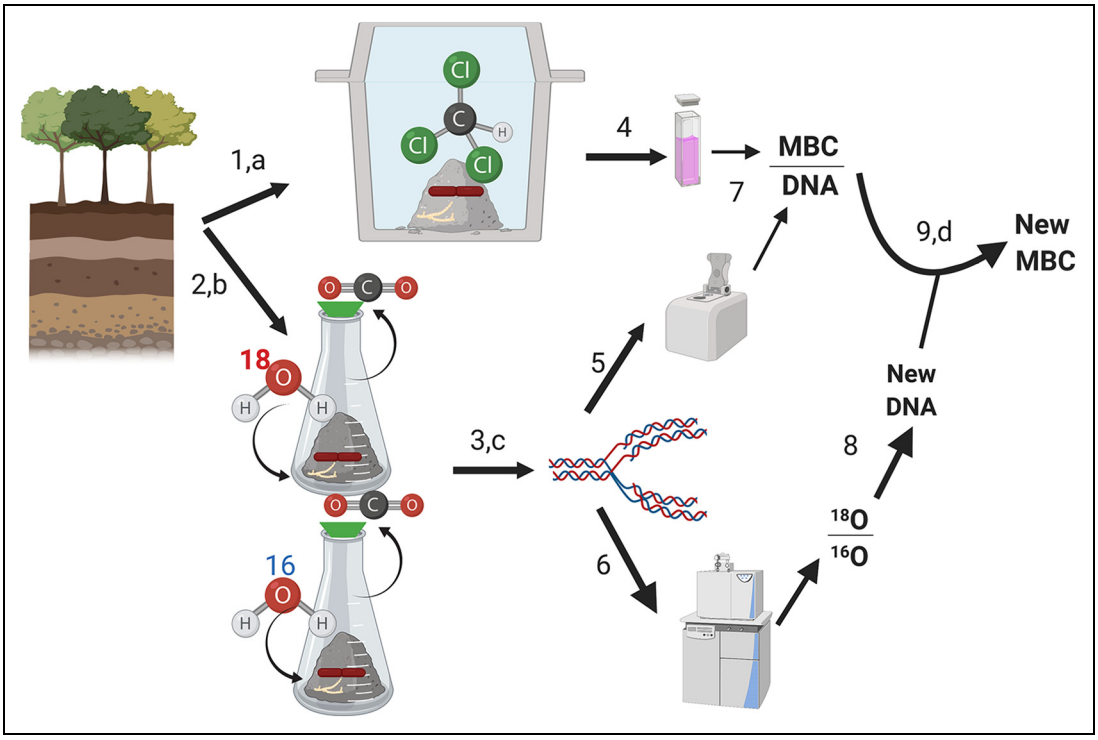

Figure 1. The ^{18}O-H_2O method of evaluating gross MBC growth (numbers), and the assumptions made (letters). Soil collected from the environment is subject to chloroform fumigation extraction (1) and incubation with ^{18}O-H_2O to determine new DNA production (2). The DNA is extracted from the soil (3). The total MBC (4) and DNA (5) are quantified. The total quantity of DNA and its ^{18}O enrichment (6) are used to determine the new DNA produced (8), and the ratio of MBC: total DNA (7) is then multiplied with this value to calculate the new MBC produced during the incubation (9). The ^{18}O-CUE method assumes efficient and unbiased extraction of MBC (a) and DNA (c); that oxygen from ^{18}O-H_2O is incorporated into new DNA to comprise a fraction of the oxygens equal to its abundance as a fraction of total soil water (b); and that the MBC: DNA ratio of the whole community represents that of the growing community (d). Figure created with BioRender.com. DOI: https://doi.org/10.1525/elementa.069.f1

Due to these known biases, there has been a recent push toward using substrate-independent measures of biomass increment, such as ^{18}O-H_2O incorporation into DNA (Blazewicz and Schwartz, 2011; **Figure 1**). For this method, soil samples are amended with ^{18}O-H_2O to 5% to 50% of the total soil water before being placed in a sealed container for 12 to 72 h (Spohn et al., 2016; Geyer et al., 2019). This duration is intended to be long enough for the incorporation of the labeled ^{18}O into DNA to be detected but short enough that the labeled ^{18}O-DNA is not recycled (Blazewicz and Schwartz, 2011). At the end of the incubation, a gas sample is taken to measure CO_2 respiration, and DNA is extracted from the incubated soil. The amount of ^{18}O incorporated into the DNA is then determined using Isotope-Ratio Mass Spectrometry (IRMS), with new DNA produced assumed to be 31% oxygen by mass (Hungate et al., 2015). Incubation with nonenriched (^{16}O) water is done in parallel to account for the natural abundance of ^{18}O in DNA. The microbial biomass carbon (MBC) is quantified using chloroform fumigation extraction on a separate sample of soil to estimate the carbon within the community pool. The MBC value is then divided by the total DNA extracted from the heavy water incubation to yield an MBC:DNA ratio for each soil sample. Multiplying the new "DNA growth" based on ^{18}O assimilation into DNA by either a sample-specific MBC:DNA ratio (Walker et al., 2018; Poeplau et al., 2019) or cross-sample average MBC:DNA ratio (Spohn et al., 2016; Geyer et al., 2019) provides an estimate of the fraction of new MBC produced during the incubation in relation to the initial MBC present in the soil sample. CUE can then be calculated as for the isotopically labeled carbon methods.

Although the ^{18}O-H_2O method is being increasingly used in empirical studies (Spohn et al., 2016a, 2016b; Walker et al., 2018; Zheng et al., 2019), its use requires making a number of untested assumptions. These assumptions include the following: DNA and MBC are completely extracted without bias (**Figure 1a and b**); extracellular water is the sole source of oxygen in DNA (**Figure 1c**); and the actively growing community is representative of the total community (**Figure 1d**). We explored the validity of these assumptions using a literature search and then used the results of this search to build and parameterize a mathematical model to assess how violating the assumptions of the ^{18}O-CUE method influences estimates of microbial growth and CUE. Finally, we applied this model to an empirical data set to evaluate the consequences of violating those assumptions in a real microbial community. Physiological differences between bacteria and fungi are relatively well studied (Rousk et al., 2008; Lennon

et al., 2012; Sun et al., 2017), and the abundance of these two groups is often determined during routine soil analyses. Therefore, our simulations center on how the sensitivity of gross MBC growth changes as a function of the fungal to bacterial fraction of soil DNA. We hypothesize that violating the assumptions required for the ^{18}O-CUE method affects estimates of the growth component of carbon use efficiency in soil.

Materials and methods

Literature review to set model parameters

We conducted a literature search to identify which assumptions of the ^{18}O-CUE method are likely to be violated and to define the range of values previously observed for such parameters (Supplementary files S1, S2). We explain those variables below before introducing the model developed to evaluate their effect on microbial growth and CUE.

The ^{18}O-CUE method relies on measurements of MBC (**Figure 1a**) and microbial DNA (**Figure 1c**) using methods known to underestimate the true values for microbes in the soil. For instance, DNA extraction yields changes depending on the method used (Martin-Laurent et al., 2001; Starke et al., 2019) and soil type (Martin-Laurent et al., 2001; Feinstein et al., 2009), with one company reporting up to 4 to 8× higher yields with its newer kit compared to its predecessor (QIAGEN Germantown, MD, USA). DNA extraction efficiency may also preferentially capture one group of taxa over another, based on properties of cell walls and cell membranes or physical location within the soil that make them more or less susceptible to cell lysis (Starke et al., 2019). There is some evidence that bacterial DNA is extracted more efficiently than fungal DNA (DNAexteffB vs. DNAexteffF, respectively; Feinstein et al., 2009; Starke et al., 2019), so we allow the values to vary separately from one another in our simulations. Likewise, MBC is extracted with different efficiencies for bacterial and fungal biomass (MBCexteffB and MBCexteffF, respectively; Jenkinson, 1976). Together, these extraction inefficiencies can cause the observed MBC:DNA ratio to deviate from the true value, with the degree of deviation potentially varying as a function of the fungal:bacterial ratio of the community.

The amount of growth we capture by measuring the amount of ^{18}O in the DNA extract may not represent the true amount of growth (**Figure 1b**). This may be for two main reasons. The first is that extracellular water is not the sole source of oxygen for DNA. Rather, anywhere from 4% to 70% of oxygen in DNA may come from metabolic water (Kreuzer-Martin et al., 2005, 2006; Li et al., 2016) or oxygenated carbon sources (Qu et al., 2020). Our model refers to this propensity toward using extracellular water or carbon sources rather than intracellular water or carbon sources as the oxygen contribution to DNA as H2Ocont, which is the fraction of extracellular water used for DNA synthesis. This value is commonly assumed to be one (i.e., 100% of new DNA oxygen is derived from extracellular water) in reported CUE values (Spohn et al., 2016a, 2016b; Geyer et al., 2019; Zheng et al., 2019), but in practice may be as low as 30% in fast-growing bacteria (Chaney et al., 1972; Hungate et al., 2015; Li et al., 2016). The second reason that not all growth is observed is "cryptic growth." One of the assumptions of the ^{18}O-CUE method is that the turnover of labeled DNA is negligible over the course of the incubation. Assuming a steady microbial community biomass, bulk turnover rates of 0.3% to 7% per day (Rousk and Bååth, 2011; Drigo et al., 2012; Spohn et al., 2016a; Gibson et al., 2018; Koch et al., 2018) indicate this expectation is reasonable because only a small fraction of DNA will turnover in the course of the incubation. However, the true DNA growth rate is likely to be higher because not all taxa (total DNA pool) are actively dividing. Estimates of the size of the nonactively dividing population vary widely, from 6% to 96% of the total community (Wang et al., 2014; Papp et al., 2018). Considering a scenario in which 96% of the community is not actively dividing leads to a 24-fold underestimation (0.96/ (1 − 0.96)) of the growth rate of the actively dividing population due to dilution with the bulk pool, but only minimal underestimation if 6% are nonactive. We use the variables activeFractionB and activeFractionF to only account for the portion of bacterial and fungal communities that are actively dividing, respectively. Factors such as predation could decrease apparent growth rate through the inefficient reallocation of labeled nucleic acids from primary to secondary consumers, particularly if predators selectively consume community members within a narrow size range (Blanc et al., 2006; Hai-Feng et al., 2014). However, we do not explicitly include this variable in our simulations below as it can in part play out through differences in activeFractionB and activeFractionF.

A final source of bias derives from differences in growth rate between bacteria and fungi that can cause the MBC:DNA ratio of the actively growing community to deviate from that of the total community (**Figure 1d**). Our model includes values for converting new DNA growth into MBC growth equivalents with group-specific conversion ratios. The growth rates of bacteria and fungi in units of µg DNA per g soil per day form the variables GRbact and GRfun, respectively. Additional variables allow for the true MBC to DNA ratio of bacteria (MBCDNAratB) to differ from that of fungi (MBCDNAratF; Mouginot et al., 2014).

Modeling sensitivity of CUE to violations of assumptions

We constructed a model and ran a set of simulations to assess the sensitivity of CUE to the methodological assumptions highlighted above. Starting with estimates of microbial growth, our work here was to reverse-simulate the range of possible true microbial biomass increment values underlying those observed values. We wished to retain the intersample differences in MBC:DNA ratio and growth and assume that estimates of the fungal:bacterial ratio are available to infer growth-related parameters. We therefore applied modifying factors to original data (see details below) using expected ratios between bacterial and fungal parameters.

To estimate the bacterial or fungal DNA fraction of biomass, we first converted the observed fungal and bacterial ribosomal gene counts (typically based on qPCR) to the expected contribution of fungal and bacterial DNA to the total DNA pool. To do this, we assumed 82 internal

Table 1. Values used to parameterize simulations of MBC growth during ^{18}O-H_2O addition to soil. See File S2 for full details. DOI: https://doi.org/10.1525/elementa.069.t1

Definition	Parameter	Value	Minimum	Maximum	Reference
Actively growing fraction bacteria	activeFractionB	0.1	0.01	1	Papp et al. (2018), Wang et al. (2014), Lennon and Jones (2011)
Actively growing fraction of fungi	activeFractionF	0.1	0.01	1	NA
Fraction of DNA oxygen from extracellular water	H2Ocont	0.7	0.3	1	Geyer et al. (2019), Hungate et al. (2015), Kreuzer-Martin et al. (2005, 2006), Li et al. (2016), Aanderud and Lennon (2011)
Bacterial DNA extraction efficiency	DNAexteffB	0.5	0.06	0.5	Feinstein et al. (2009), personal obs
Fungal DNA extraction efficiency	DNAexteffF	0.33	0.04	0.5	Feinstein et al. (2009), personal obs
Bacterial growth rate (daily multiplier)	GRbact	0.167	0.003	3.4	Gibson et al. (2018), Koch et al. (2018), Rousk and Bååth (2011), Blazewicz et al. (2014), Soares and Rousk (2019)
Fungal growth rate (daily multiplier)	GRfun	0.0088	0.0001	0.8	Drigo et al. (2012), Rousk and Bååth (2011), Blazewicz et al. (2014), Soares and Rousk (2019), Eric Morrison, pers. comm.
MBC:DNA ratio of bacteria	MBCDNAratB	13.22	3.6	37	Mouginot et al. (2014), Christensen et al. (1993, 1995), Portillo et al. (2013), Soares and Rousk (2019), Bakken and Olsen (1989), Kristiansen et al. (2012), Makino et al. (2003)
MBC:DNA ratio of fungi	MBCDNAratF	1,070	90	3,300	Mouginot et al. (2014), Anderson and Martens (2013), Kristiansen et al. (2012), Grimmett et al. (2013), Leckie et al. (2004)
Bacterial MBC extraction efficiency	MBCexteffB	0.32	0.1	0.51	Jenkinson (1976), Dictor et al. (1998), Tate et al. (1988), Joergensen (1996)
Fungal MBC extraction efficiency	MBCexteffF	0.33	0.21	0.45	Dictor et al. (1998), Tate et al. (1988), Joergensen (1996), Martin et al. (1959)
Bacterial 16S copies per genome	rrnpergenome	2.25	1	15	DeAngelis et al. (2015), Stoddard et al. (2015)
Fungal ITS copies per genome	ITSpergenome	82	14	1,442	Lofgren et al. (2019)

transcribed spacer copies per genome (ITSpergenome) and a median genome size of 5×10^8 bp for fungi (Chen et al., 2017; Lofgren et al., 2019), and 2.25 16S copies per genome (rrnpergenome) and a genome size of 5×10^6 bp for bacteria (DeAngelis et al., 2015; Chen et al., 2017; **Table 1**). To get the true fungal fraction of DNA (Ffrac), we used the observed ITS copies (copiesITS) and 16S ribosomal RNA copies (copies16S) from qPCR. We first accounted for inefficiencies in DNA extraction efficiency for bacteria (DNAexteffB) and fungi (DNAexteffF) and converted to bacterial genome counts (genomes16S) and fungal genome copies (genomesITS) as follows:

$$\text{genomesITS} = \frac{\frac{1}{\text{DNAexteffF}} \times \text{copiesITS}}{\text{ITSpergenome}} \quad (1)$$

$$\text{genomes16S} = \frac{\frac{1}{\text{DNAexteffB}} \times \text{copies16S}}{\text{rrnpergenome}} \quad (2)$$

And then convert the fungal and bacterial genome copies to DNA mass per gram of soil for fungi (FDNA) and bacteria (BDNA) as follows:

$$\text{FDNA} = \frac{\text{genomesITS} \times 5 \times 10^8 \times 650 \times 10^6}{6.02214 \times 10^{23}} \quad (3)$$

$$\text{BDNA} = \frac{\text{genomes16S} \times 5 \times 10^6 \times 650 \times 10^6}{6.02214 \times 10^{23}} \quad (4)$$

Where 650×10^6 is the molecular weight of the average DNA base pair in μg mole^{-1} and 6.02214×10^{23} is Avogadro's constant. The corresponding ratio of fungus DNA:total DNA (Ffrac) corrected for extraction efficiency and genome size is then:

$$\text{Ffrac} = \frac{\text{FDNA}}{\text{FDNA} + \text{BDNA}} \quad (5)$$

The corresponding corrected total DNA (totalDNAActual) in the combined actively growing and nongrowing populations' DNA pool used for MBC:DNA ratio calculation is:

$$\text{totalDNAActual} = \text{totalDNA} \times \text{Ffrac} \times \frac{1}{\text{DNAexteffF}} + \text{totalDNA} \times (1 - \text{Ffrac}) \times \frac{1}{\text{DNAexteffB}} \quad (6)$$

We can then calculate relative contributions to the MBC pool for fungi (fcont) and bacteria (bcont), by combining values for the ratio of MBCDNAratF and MBCDNAratB, bacterial and fungal DNA extraction efficiencies (MBCexteffB and MBCexteffF), and the fraction of DNA that is fungal (Ffrac).

$$\text{fcont} = \frac{\text{MBCDNAratF} \times \text{MBCexteffF} \times \text{Ffrac}}{\text{MBCDNAratF} \times \text{MBCexteffF} \times \text{Ffrac} + \text{MBCDNAratB} \times \text{MBCexrteffB} \times (1 - \text{Ffrac})} \quad (7)$$

$$\text{bcont} = 1 - \text{fcont} \quad (8)$$

These values can then be used to calculate the actual amount of MBC (MBCactual) and the MBC:DNA ratios for fungi (FMBCDNAratio) and bacteria (BMBCDNAratio) as follows:

$$\text{MBCactual} = \frac{\text{fcont} \times \text{MBCobs}}{\text{MBCexteffF}} + \frac{\text{bcont} \times \text{MBCobs}}{\text{MBCexteffB}} \quad (9)$$

$$\text{MBCDNAactual} = \frac{\text{MBCactual}}{\text{totalDNAActual}} \quad (10)$$

$$\text{FMBCDNAratio} = \frac{\frac{\text{fcont} \times \text{MBCobs}}{\text{MBCexteffF}}}{\frac{\text{totalDNA} \times \text{Ffrac}}{\text{DNAexteffF}}} \quad (11)$$

$$\text{BMBCDNAratio} = \frac{\frac{\text{bcont} \times \text{MBCobs}}{\text{MBCexteffB}}}{\frac{\text{totalDNA} \times (1 - \text{Ffrac})}{\text{DNAexteffB}}} \quad (12)$$

Now we calculate the Ffrac_active, which is the fraction of new growth attributed to fungi during the incubation. It is a function of the relative growth rates of bacteria (GRbact) and fungi (GRfun), as well as the Ffrac in the starting bulk community and the fraction of the cells which are actively growing (activeFractionF and activeFractionB), rather than dormant.

$$\text{Ffrac\_active} = \frac{\text{Ffrac} \times \text{activeFractionF} \times \text{GRfun}}{\text{Ffrac} \times \text{activeFractionF} \times \text{GRfun} + (1 - \text{Ffrac}) \times \text{activeFractionB} \times \text{GRbact}} \quad (13)$$

We then accounted for DNA extraction efficiency of fungi (DNAexteffF) and bacteria (DNAexteffB), as well as the use of intracellular water and/or other sources of DNA oxygen (H2Ocont) for the growing community:

$$\text{fungGrowthact} = \frac{\text{NewDNAobs} \times \text{Ffrac}_{\text{active}}}{\text{H2Ocont} \times \text{DNAexteffF}} \quad (14)$$

$$\text{bactGrowthact} = \frac{\text{NewDNAobs} \times (1 - \text{Ffrac}_{\text{active}})}{\text{H2Ocont} \times \text{DNAexteffB}} \quad (15)$$

Finally, we converted these DNA growth estimates to the gross MBC growth which occurred after applying our methodological corrections (MBCgrowthactual) and calculated true CUE ($\text{CUE}_{\text{actual}}$ using the observed respiration rate (per day):

$$\text{MBCgrowthactual} = \text{fungalGrowthact} \times \text{FMBCDNAratio} + \text{bactGrowthact} \times \text{BMBCDNAratio} \quad (16)$$

$$\text{CUE}_{\text{actual}} = \frac{\text{MBCgrowthActual}}{\text{MBCgrowthActual} + \text{respiration}} \quad (17)$$

Effect of common experimental manipulations on microbial growth estimates

Since fungi are estimated to contribute much less to the total DNA pool in our microcosms compared to some field sites (2.3% on average vs. 50% or more; Baldrian et al., 2012), we completed additional simulations which would allow us to explore how violating these assumptions could influence estimates of microbial growth considering a higher degree of fungal dominance. Common experimental manipulations such as antibiotics, organic, or inorganic substrate addition and changes in temperature and moisture may deferentially affect the parameters of our model, so we used these simulations to infer how microbial growth may be misestimated under different fungal abundance. The full equations for this are in supplementary file S1.

Empirical application of model

We used a subset of data from a previously completed soil microbial diversity manipulation experiment to explore how altered fungal to bacterial ratios influence estimates of CUE (Domeignoz-Horta et al., 2020) under a range of methodological assumptions. Microbial communities were extracted from temperate deciduous forest soil and either the complete ("fungi + bacteria") or filtered to less than 0.8 μM fraction ("bacteria-only") was used to inoculate a model soil system. Each microcosm consisted of a 60 ml glass tube with 20 g of an artificial soil containing 70% acid-washed play sand, 20% ashed and acid-washed silt, and 10% homoionic calcium bentonite clay. The communities were grown for 4 months, with weekly additions of 0.5 mg g soil^{-1} cellobiose and 0.05 mg g soil^{-1} ammonium nitrate solutions as sources of C and N, respectively. Soils were kept at 25°C and 60% water holding capacity throughout (Howard and Howard, 1993). At the end of the 4-month incubation, MBC was measured using direct chloroform extraction (CFE) on three paired 1.5 g soil

subsamples (Setia et al., 2012) followed by quantification using colorimetry (Bartlett and Ross, 1988). The experiment consisted of 12 replicates each for "fungi + bacteria" and "bacteria-only" microcosms. We excluded two "bacteria-only" microcosms where MBC was below the detection level.

CUE was measured by adding ^{18}O-H_2O to two 0.32 g soil subsamples of the soil at a final concentration of 20 atom %, keeping the soil at 60% water holding capacity. Controls for background heavy oxygen incorporation were generated by adding ^{16}O-H_2O in place of heavy water to a third soil subsample. The samples and controls were then placed in sealed tubes for 24 h, and the CO_2 produced during this time was measured using a Quantek instruments model 906 IRGA. The soil samples were stored at –80°C until DNA extraction using the Qiagen Powersoil HTP kit. The resultant DNA was quantified using PicoGreen (Invitrogen), and its ^{18}O enrichment was measured using IRMS at the UC Davis Stable Isotope Facility. CUE was calculated as per Spohn et al. (2016), except that we used a sample-specific MBC:DNA ratio and a *t*-test was used to evaluate whether CUE differed between the two microbial community types.

The abundances of total bacteria and total fungi were assessed by real-time quantitative PCR (qPCR) using 16S rRNA primers (Muyzer et al., 1993) and ITS primers (Fierer et al., 2005), respectively. The abundance in each soil sample was based on increasing fluorescence intensity of the SYBR Green dye during amplification against standards of *Saccharomyces cerevisiae* for fungi and *Rhizobium* species AN6A (IMG taxon ID 2619618868) for bacteria. An inhibition test performed by running serial dilutions of DNA extractions prior to qPCR did not detect amplification inhibition. The qPCR assay was carried out in a 15-µl reaction volume containing 2 ng of DNA, 7.5 µl of mastermix (QuantiFast SYBR Green PCR Master Mix), and each primer at 1 µM. The 16S qPCR conditions were: 15 min at 95°C; 40 × 15s at 94°C, 30s at 55°C, 30s at 72°C; and a melting curve. ITS qPCR conditions were: 15 min at 95°C; 40 × 15s at 94°C, 30s at 46°C, 30s at 72°C; and a melting curve. These values were corrected to a genome counts basis using median values for ITS copies from Lofgren et al. (2019) and median values for bacterial 16S ribosomal RNA operon copy number from Stoddard et al. (2015). Two independent qPCR assays were performed for each gene, and the qPCR efficiencies for both genes ranged between 85% and 102%. The ITS and 16S rrN copy number, respiration rate, and observed MBC and DNA pools from these microcosms were subsequently used as inputs to the mathematical model described above. Please note that fungal removal treatment was incomplete in 2 of the 10 "bacteria-only" microcosms, and we refer to the microcosms by the treatment rather than the actual outcome of the treatment in our results and discussion below.

Calculations

All analyses were completed in R v3.4.0 (R Core Team, 2016), and results were plotted using ggplot2 (Wickham, 2009). We used both the MBCgrowthactual and CUEactual in our analysis. The former allows us to see how the growth component of CUE may differ from the value we observed, while the latter allows us to see how much misestimates of growth influence CUE once the carbon allocated to respiration is also accounted for. We completed our simulations by setting one parameter to either its highest or its lowest value observed in the literature (**Table 1**), keeping all remaining parameters at their best estimated values. This generated a simulated growth and CUE for each soil sample for every parameter combination. After calculating the cross-sample median and standard error for each parameter combination and community type, we compared the log ratio of simulated to observed MBC growth for each soil sample. We used the ratio of simulated to observed MBC growth because the absolute amount of observed growth was widely different across soil samples. All values were log transformed, so equivalent increases and decreases in growth received the same weighting in our calculations.

We also calculated a sensitivity score to identify those parameters where a small amount of uncertainty is expected to lead to a disproportionately large change in simulated CUE. Sensitivity values (a.k.a. "parameter-standardized deviations") were calculated as per Allison et al. (2010):

$$\text{Sensitivity} = \frac{|\log10(\text{highoutput}) - \log10(\text{lowoutput})|}{|\log10(\text{highparameter}) - \log10(\text{lowparameter})|}, \quad (18)$$

where *high output* is the observed CUE under the high parameter value, and *low output* is the observed CUE under the low parameter value. "High parameter" and "low parameter" correspond to the value used for the parameter of interest in the corresponding simulations and were derived based on a literature search as described in **Table 1** and the section below.

Results and discussion

Literature review of sources of ^{18}O biases

The actively growing community assayed during ^{18}O-H_2O-based growth measurements is unlikely to be representative of the total community. In particular, mean MBC:DNA ratio, growth rate, and ribosomal RNA operon copy number per genome may differ between bacteria and fungi by an order of magnitude or more (**Table 1**). The degree to which growth estimates will be affected by deviating from assumptions of the ^{18}O-H_2O-method will depend mostly on the fungal:bacterial ratio of the microbial community.

The consequences of violating ^{18}O-CUE method assumptions for growth estimates depends on fungal dominance

Our simulations predicted that observed CUE generally underestimated the true CUE value. Whereas observations assumed that 100% of the oxygen in new DNA came from extracellular water (Spohn et al., 2016b; Zheng et al., 2019), our simulations assumed that only 70% of the new DNA oxygen did. This led to reduced estimates of CUE and growth in communities both with and without fungal removal (**Figure 2a and b**). However, the responsiveness

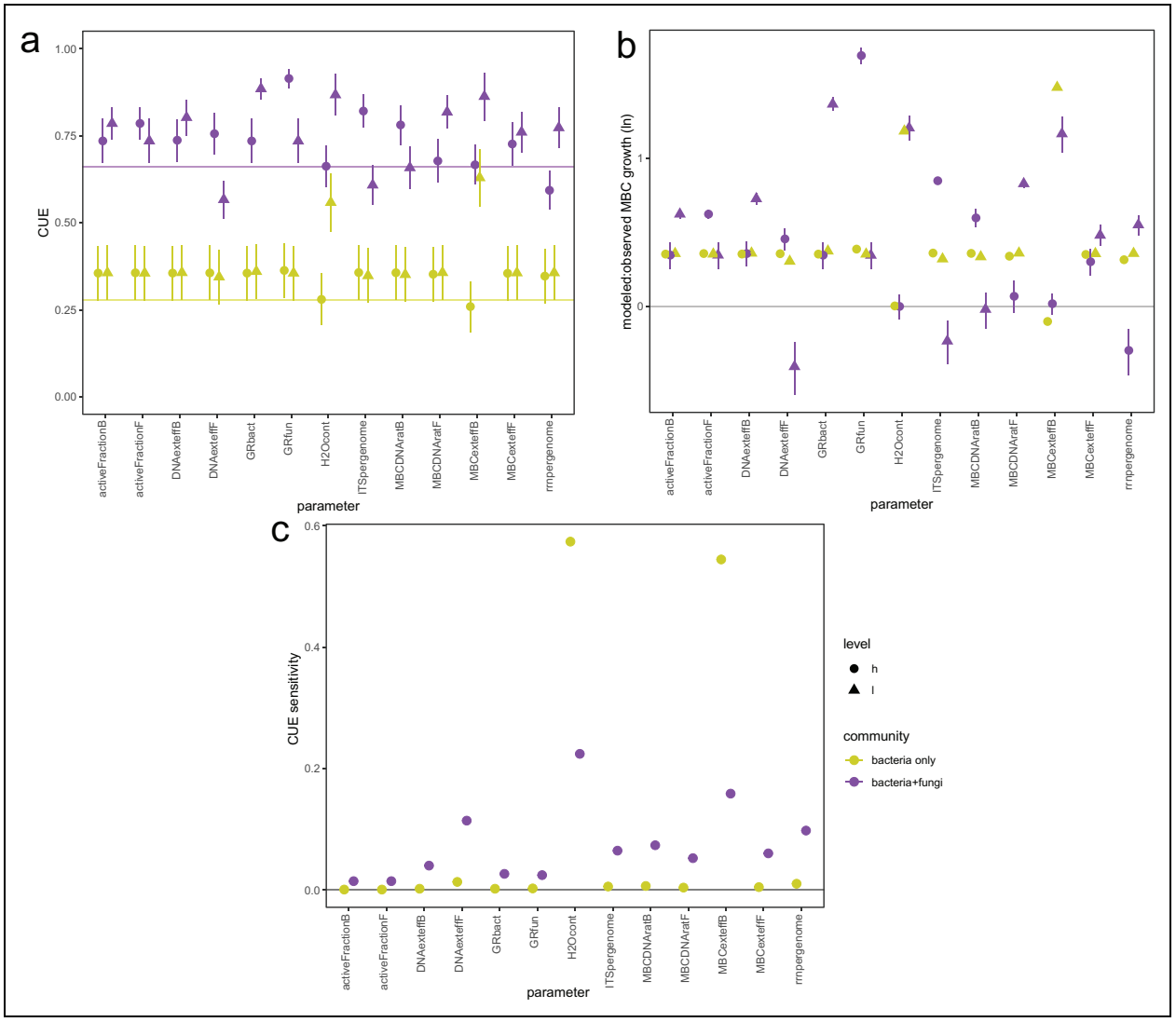

Figure 2. CUE (a, c) and microbial growth (b) of microcosms inoculated with "bacteria-only" (<0.8 µm fraction of soil community; $n = 10$) or "bacteria +fungi" communities ("bacteria and fungi"; $n = 12$), under various methodological assumptions. The x-axis denotes which parameter was tested, and shape denotes whether the high ("h") or low ("l") limit derived from the literature in **table 1** was used for the parameter of interest. Color denotes the community. Error bars denote standard error of the mean. (a) Simulated CUE values are plotted with a horizontal line marking the median observed CUE for the corresponding community type. (b) The log ratio of simulated to observed values for the growth component of CUE is plotted with the horizontal line indicating no effect of changing the parameter. (c) Relative sensitivity of CUE estimates to changes in parameters, presented as the deviation between the observed and simulated CUE values. DOI: https://doi.org/10.1525/elementa.069.f2

of CUE estimates to changes in parameters varied in a community-dependent manner.

Estimates of growth were more responsive to changes in the parameters than CUE, and estimates of both growth and CUE were more sensitive to changes in parameters in "bacteria + fungi" microcosms compared to those with "bacteria alone." Growth was underestimated by up to 5.4×, while CUE was only underestimated by up to 2.3× (**Figure 2a and b**). This can be attributed to respiration offsetting some of the responsiveness in the growth component of CUE. Here we will further discuss CUE as it is typically the ultimate metric of interest with the ^{18}O-CUE method. Lower responsiveness of CUE in "bacteria-only" microcosms compared to "bacteria + fungi" microcosms can be attributed to the near absence of fungi in the former. As a result of that, the characteristics of the community which actively grew during the simulations were more representative of the total community used in MBC:DNA calculations. On the other hand, more of the differences in fungal and bacterial parameters were able to play out in communities where both bacteria and fungi were present. Here we explore the effect of variation in some of the parameters with the greatest influences on CUE estimates.

Differences in both the DNA extraction efficiency and ribosomal copies per genome across soil microbial groups can lead to under- and overestimation of CUE within our simulation framework. If the DNA extraction efficiency of

fungi was low, the MBC:DNA ratio used for converting the new DNA to gross MBC growth was higher than it should have been, and the observed CUE resulted higher than the true CUE (**Figure 2a**). Similarly, decreasing the mean fungal ITS copies per genome or increasing the mean bacterial rrN per genome resulted in an overestimation of CUE by decreasing the bacterial in relation to the fungal contribution to the total DNA pool. Given the relatively short incubation times of these microcosms, the relatively high rates of substrate input, and the observation that CUE was greater than the proposed upper limit of 80% under slow bacterial growth (Gommers et al., 1988; **Figure 2a**), it is likely that fast-growing bacteria with high rrN (Stevenson et al., 2004) dominated these communities, and so CUE could have been overestimated in fungi+bacteria microcosms. When the growing bacterial community has a high rrN per genome, it contributes relatively less to the total DNA pool, and so the MBC:DNA ratio more closely represents the high value typical of fungi than the lower value typical of bacteria (**Table 1**). As a result, the new DNA growth is multiplied by an MBC:DNA ratio which is too high, leading to the overestimation of both growth and CUE.

One of the first improvements to the ^{18}O-H_2O CUE method was the introduction of sample-specific MBC:DNA conversion factors (Spohn et al., 2016a, 2016b; Geyer et al., 2019). However, our results show that this modification does not improve the accuracy of CUE measurements unless the MBC:DNA ratio of the total community is representative of the actively growing community. In our simulations, we allowed this to manifest as differences in the growth rates, extraction efficiencies, and "true" MBC:DNA ratios of bacteria and fungi, but there are numerous other axes not captured here which may be important.

One such axis is biological differences within bacteria, for instance, in growth rates and biomolecule extraction efficiency. For instance, the MBC:DNA ratio of the "bacteria-only" microcosms was very low, sometimes below 1, suggesting that the bacterial biomass carbon might have been inefficiently extracted. By contrast, observed MBC:DNA ratios of natural soil communities generally fall between 3 and 60 (Anderson and Martens, 2013; Spohn et al., 2016b), with values as low as 3.6 for bacteria and as high as 3,300 for filamentous fungi in the lab (Supplementary file S2). These low MBC:DNA ratios may be either biological or technical in origin. From a biological perspective, the true MBC:DNA ratio of bacteria is lower for small, slow-growing, and starving or oligotrophic cells (Christensen et al., 1993, 1995; Lever et al., 2015). Over the course of the 4-month incubation, less respiration—and presumably less growth—was detected in the bacteria-only microcosms, which could indicate these cells were smaller. Technical factors also make an accurate estimation of the MBC:DNA ratio challenging and may have biased our results toward high ratios in more fungally dominated communities. For instance, small cells have a large amount of membrane (which MBC extraction methods do not effectively capture; Dictor et al., 1998; Jenkinson, 1976) relative to the cytoplasm (which it does), therefore exacerbating the genuinely lower MBC:DNA ratio of these cells. Chloroform-lysed microbial biomass components such as DNA and proteins also have a high affinity for clay surfaces (Kleber et al., 2007). If it were the case that there was reduced microbial growth in the "bacteria-only" microcosms, less necromass would have been generated during the incubation. Thus, more bare mineral surfaces could be available for carbonaceous protein-rich biomass components to stick to during CFE in the bacteria-only compared to bacteria+fungi microcosms. This would reduce MBC extraction efficiency in the former compared to the latter and may also apply to studies where soils with different clay contents (C sorption potentials Abramoff et al., 2020) are used (Zheng et al., 2019). While our conclusion of reduced CUE with fungal removal held within the constraints of our simulation scenarios, our work clearly still leaves room for exploration of additional factors that may diminish the magnitude of the fungal removal effect observed in our wet lab experiment. Some of these variables may unintentionally be impacted by experimental manipulations in other studies, so we next highlight how some of these artifacts may arise.

Effect of common experimental factors on apparent microbial growth

Researchers are commonly interested in how various abiotic and biotic factors influence microbial physiology. In applying experimental manipulations, researchers may unintentionally induce inaccurate estimates of growth, as some of the assumptions of the ^{18}O-H_2O method are violated (**Table 2**). How these manipulations affect estimates growth is expected to depend on the fungal to bacterial ratio of the microbial community present.

One commonly manipulated factor is temperature. CUE has been proposed to change in response to temperature (Bölscher et al., 2020), but applying distinct temperatures will simultaneously change the sources of oxygen to DNA (Blake et al., 2016) and the relative growth rates of bacteria and fungi (Pietikäinen et al., 2005). Using simulations where the true growth was a priori known, we found that comparing soils incubated under different temperatures could lead to a 15-fold underestimation to 2-fold overestimation of growth in a bacteria-dominated community (90% of community DNA is bacterial). On the other hand, there could be anywhere from an 8-fold underestimation to 10-fold overestimation of growth in a community characterized by an equal distribution of bacterial and fungal DNA in the total community DNA (**Table 2**). Therefore, researchers measuring microbial growth at different temperatures should both generate an estimate of fungi:bacteria fraction and consider different growth rate responses of bacteria versus fungi when interpreting their results.

Not all experimental manipulations are expected to cause such large uncertainty in microbial growth. For example, inorganic nutrient (e.g., nitrogen or phosphorus) addition could lead to much smaller deviations between the observed and expected gross MBC growth values if compared to organic substrate addition. Under this

Table 2. Effect of various possible experimental manipulations and potential fold deviations between observed and simulated gross microbial growth. DOI: https://doi.org/10.1525/elementa.069.t2

Experimental manipulation	Parameters affected	Ffrac = 0.1	Ffrac = 0.5
Antibiotic/inhibitor addition	activeFractionB, activeFractionF	−2.6 to +2	−2.8 to +8
Substrate addition	activeFractionB, activeFractionF, H2Ocont, GRfun, Grbact	−15.2 to +2.1	−8.2 to 10.9
Drying/rewetting soils	activeFractionB, activeFractionF, H2Ocont, DNAexteffF, DNAexteffB, MBCexteffF, MBCexteffB	−2.6 to +2.1	−2.8 to +8
Changing temperature	H2Ocont, GRbact, GRfun	−15.2 to +2.1	−8.2 to +10.9
Inorganic nutrient addition	MBCDNAratB, MBCDNAratF	−2.8 to +2.7	−1.2 to +2.6

Note. Positive values indicate fold increases, while negative values indicate fold decreases. Code for fixed fungal fraction (FFrac) of 0.1 and 0.5 simulations can be found in supplement 1.

scenario, we assume that C or energy rather than N or P are the primary limiting nutrients for microbes (Hobbie and Hobbie, 2013), and so inorganic nutrient addition is not expected to substantially change growth rate in the way that organic C addition is. Because CUE estimates are more sensitive to relative growth rate, it is the proposed differential effect of organic substrate addition on the relative growth rate of bacteria and fungi which drives this greater sensitivity. In this case, knowing the fungal DNA fraction is less important, but knowing how nutrient addition may change the relationship between cell carbon content and DNA production would help refine estimates of growth. It is also important to note that recent work has been completed to try and constrain the contribution of extracellular water to new DNA growth in soil (Qu et al., 2020) and to develop new vapor-based CUE measurements which disrupt the soil moisture regime less (Canarini et al., 2020). Implementing these methodological changes to the ^{18}O water method will effectively reduce the range of physiological parameters causing the observed CUE to deviate from its true value.

Shortcomings

There are a number of variables that may affect the accuracy of CUE and growth estimates which are not captured in our simulation framework. First, many values used to parameterize the CUE simulations are based on isolates grown in the lab under ideal conditions. However, microbes are known to grow very differently in the lab compared to in soil. For instance, well-fed bacterial cultures will have lower dormancy and less starvation-induced reductive cell division than those found in soil (Lever et al., 2015). Cultivation bias toward fast-growing organisms only exacerbates this, as the ratio of cytoplasm C which can be measured by chloroform fumigation extraction to cell wall C (which cannot be measured by CFE) will be greater in the copiotrophic organisms we tend to study in the lab (Portillo et al., 2013). The DNA:MBC ratio has been observed to be higher in small, slow-growing cells in communities extracted from soil (Christensen et al., 1995) but remains constant over a wide range of growth rates in *Escherichia coli* (Donachie, 1968; Kubitschek, 1974). Given how poorly defined this relationship is, we did not include it as a component in our simulations.

Furthermore, we note that the contribution of intracellular water to DNA backbone oxygen was 70% for fast- compared to ∼4% in slow-growing bacterial culture on rich media (Kreuzer-Martin et al., 2005, 2006; Li et al., 2016), with the contribution of growth substrate oxygen also varying depending on substrate chemistry and the metabolic status of the organisms (Berg et al., 2002a, 2002b). Therefore, it is likely important to account for inter-sample differences in the contribution of ^{18}O-H_2O to DNA oxygen as a function of growth rate. However, in the absence of knowledge about where bacterial and fungal growth in soil fit on this alternative water source spectrum, we did not include this parameter in our simulations. Comparing CUE in soils with very different substrate types or communities should also be done with great care because substrates differ in the degree to which they can contribute oxygen to DNA (Qu et al., 2020), and biosynthetic pathways differ in the oxygen sources they preferentially assimilate into bases (Berg et al., 2002b, 2002c). A recent study concluded that glucose contributes 10× fewer oxygen to DNA than extracellular water does (Qu et al., 2020) but did not address how this may vary according to glucose supply rate and failed to account for contributions of oxygen from SOM. As such, the relative importance of these substrate-by-community metabolic interactions remains poorly quantified, and a single number is unlikely to successfully correct for alternative oxygen sources to DNA.

Finally, determining the true contribution of different groups of microbes to the soil DNA pool remains challenging. Accurate predictions based on metagenomes are limited by both database biases and the abundance of noncoding DNA in eukaryote genomes, while imperfect primers and differences in ribosomal RNA operon copy number limit the utility of qPCR. Since our simulations assume that fungi and bacteria have fundamentally different traits, accurate estimates of MBC growth in our framework will always be limited by how well fungal dominance is quantified.

Conclusion

CUE is an essential descriptor of soil carbon cycling, with important ramifications for both the ecology and

biogeochemistry of soil. The need to measure this parameter is matched with a growing awareness of the various shortcomings in its quantification. Here we examined how assumptions associated with this method affect the conclusions drawn from its estimation. We found that the common assumption that all new DNA oxygen comes from extracellular water is likely leading to a consistent underestimation of microbial growth. However, accounting for alternative oxygen sources in growth estimates is not straightforward because extracellular water oxygen use is itself sensitive to environmental conditions. Parameters that are weakly constrained due to technical and/or biological factors such as fungal and bacterial MBC:DNA ratio and biomolecule extraction efficiency led to a wide range of simulated growth rates. However, the absolute influence of these variables depends on the fungal to bacterial ratio, indicating that the parameters that growth estimates are most sensitive to will diverge between soils with different microbial communities. Common manipulations can influence growth estimates, and our simulations offer a framework for future studies to evaluate how their experimental conditions may affect its growth estimates.

Data Accessibility Statement

The data and scripts used in this manuscript can be found in OSF at https://osf.io/xcdk6/?view_only=05db1bb33a03423d9aa5cc72ef298476.

Supplemental files

The supplemental files for this article can be found as follows:

- Supplementary file S1.

  Description of equations and parameters used to evaluate how estimates of MBC growth from the ^{18}O-H_2O method may be affected by various experimental manipulations.

- Supplementary file S2.

  Parameter values resulting from a literature search for variables used in microbial carbon growth and CUE simulations.

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Competing interests

The authors declare no competing interests in the execution or publication of this research.

Author contributions

GP conceived the project, designed the simulations and completed the analysis. GP and KMD completed the literature search. LDH completed the microcosm experiment. All authors participated in drafting and revising the manuscript.

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