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Beyond growth: the significance of non-growth anabolism for microbial carbon-use efficiency in the light of soil carbon stabilisation

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Abstract

Microbial carbon-use efficiency (CUE) in soils captures carbon (C) partitioning between anabolic biosynthesis of microbial metabolites and catabolic C emissions (i.e. respiratory C waste). The use of C for biosynthesis provides a potential for the accumulation of microbial metabolic residues in soil. Recognized as a crucial control in C cycling, microbial CUE is implemented in the majority of soil C models. Due to the models' high sensitivity to CUE, reliable soil C projections demand accurate CUE quantifications. Current measurements of CUE neglect microbial non-growth metabolites, such as extracellular polymeric substances (EPS) or exoenzymes, although they remain in soil and could be quantitatively important. Here, we highlight that disregarding non-growth anabolism can lead to severe underestimations of CUE. Based on two case studies, we demonstrate that neglecting exoenzyme and EPS production underestimates CUE by more than 100% and up to 30%, respectively. Using these values in model simulations, we observed that the model projects up to 34% larger SOC stocks when non-growth metabolites are considered for estimating CUE. Our considerations outlined here challenge the current ways how CUE is measured. Research efforts should focus on (i) advancing CUE estimations by capturing the multitude of microbial C uses, (ii) improving techniques to quantify non-growth metabolic products in soil, and (iii) providing an understanding of dynamic metabolic C uses under different environmental conditions and over time. In the light of current discussion on soil C stabilization mechanisms, we call for efforts to open the ‘black box’ of microbial physiology in soil and to incorporate all quantitative important C uses in CUE measurements.
1. Introduction

The microbial origin of stabilized soil organic C (SOC) has received increasing attention in recent years (e.g. Domeignoz-Horta et al., 2021; Kallenbach et al., 2016, 2015; Liang et al., 2020, 2017; Miltner et al., 2012). To date, it remains challenging to quantify the contribution of microbial-derived C to stable SOC (Liang et al., 2019), but some findings suggest that microbial-derived C may make up a quarter to more than half of total SOC (Deng and Liang, 2021; Liang et al., 2019; Miltner et al., 2012). Despite quantitative uncertainties concerning microbial-derived stable SOC, the microbial metabolic performance is a key factor in soil C dynamics, because the vast majority of organic C inputs to soil will be eventually processed by soil microorganisms.

Soil C inputs will thus largely be subjected to microbial C allocation towards catabolic C emissions (i.e. C waste via respiration) or biosynthesis, with the latter leading to C remaining in soil. Recognized as a crucial control in C cycling, this microbial carbon use efficiency (CUE) is implemented, implicitly or explicitly, in all soil C models (Schimel et al., 2022; Schimel, 2013), which respond highly sensitive to even small changes in CUE (Allison et al., 2010; Bölscher et al., 2020; Frey et al., 2013; Hyvönen et al., 1998). Due to the models’ high sensitivity, reliable SOC projections require accurate CUE quantifications, capturing all metabolic C uses within microorganisms.

The concept of microbial CUE—as applied in soil ecology—neglects considerable parts of the microbially processed C, because current methods focus on capturing growth/biomass increases and assume non-growth anabolism as quantitatively unimportant (Manzoni et al., 2018; Paul and Clark, 1989). Here, we show that this quantitatively neglected C will affect CUE estimations. The neglected C comprises all extracellular metabolites released from microbial cells into the surrounding soil and potentially intracellular non-growth metabolites (Fig. 1). Which anabolic C
uses are exactly ignored depends on definitions and methods applied to measure CUE. Despite its key importance for soil C cycling, CUE remains an ambiguous and poorly defined concept (Schimel et al., 2022). Within soil ecology, two partially different notions of CUE have emerged, the substrate-specific CUE, which measures the incorporation of C-isotope labels into microbial biomass (Geyer et al., 2019; Manzoni et al., 2012; Steinweg et al., 2008), and the substrate-independent CUE, measuring growth via $^{18}$O-water incorporation into DNA (Geyer et al., 2019, 2016; Spohn et al., 2016). While substrate-specific CUE treats all C incorporated into microbial biomass as efficiently used (and thus remaining in soil when implemented in soil C models), substrate-independent CUE considers only C directed towards growth as efficiently used C. Both CUE methods and underlying concepts neglect microbial non-growth anabolism. Non-growth anabolism includes all extracellular metabolites, such as extracellular polymeric substances (EPS), exoenzymes or nutrient mobilizing compounds (Flemming and Wingender, 2010; Van Bodegom, 2007), and certain intracellular metabolites, such as storage compounds or endoenzymes (Mason-Jones et al., 2023, 2022). Yet, C directed towards the synthesis of such compounds is not ‘inefficiently’ used C, as it serves crucial functions supporting microorganisms to survive and is primordial for microbial life itself. More importantly, this C remains in soil and thus provides a potential for long-term C stabilization. When CUE, based on current methods, is implemented in soil C models, the models paradoxically treat non-growth metabolites as emitted C waste leaving the soil. Neglecting microbial non-growth anabolism may introduce a bias when quantifying microbial CUE.

Here, we (i) scrutinize the idea that non-growth anabolism can be ignored for CUE investigations, (ii) suggest adjustments to common CUE approaches, (iii) demonstrate that current assessments of CUE measure only an ‘apparent’ CUE which can significantly
underestimate ‘actual’ CUE, (iv) illustrate the potential consequences for SOC projections and 
(v) outline research needs and potential ways forward.

2. Why is non-growth anabolism disregarded for microbial CUE?

Why do current concepts of microbial CUE in soil ecology neglect non-growth anabolism, 
despite the respective C remains in soil and serves important microbial survival strategies? The 
reason may be three-fold: (i) Ideas of CUE evolved parallel in various scientific fields, 
contributing to the concept’s ambiguity and amorphous definition (Geyer et al., 2016; Manzoni 
et al., 2018; Schimel et al., 2022); (ii) Non-growth anabolism is considered quantitatively 
egligible compared to C used for growth (Manzoni et al., 2018); and (iii) Quantification of 
microbial non-growth anabolism in soil is challenging:

(i) Concepts of CUE have been developed in various subfields of biology. While addressing 
roughly the same idea, specific definitions and conceptualizations of CUE diverge (Geyer et al., 
2016; Manzoni et al., 2018). The assignment of C as efficiently used can comprise growth, 
biomass production or entire biosynthesis (Manzoni et al., 2018, 2012). Depending on the 
organism and specific situation, these three entities can be almost similar or they differ 
substantially (Manzoni et al., 2018). The concept of microbial CUE in soil ecology has been 
largely influenced by the idea of microbial-growth efficiency (often called growth yield; see 
Supplementary Note for discussion on how neglecting non-growth anabolism affects estimations 
of growth efficiency). The latter is commonly used in microbiology (e.g. Gommers et al., 1988; 
Linton, 1991; von Stockar and Marison, 1993) and has influenced the CUE concept in soil 
ecology (Frey et al., 2001; Herron et al., 2009; Sinsabaugh et al., 2013; Spohn et al., 2016; Thiet 
et al., 2006). In microbiology, growth efficiencies are, however, frequently measured in pure 
cultures with (near)optimal conditions for microbial growth. Under such conditions, non-growth
anabolism (e.g. EPS, storage compound or osmolyte production) is less important for microbial survival than in harsh soil environments. As such, pure culture studies serve purposes that are often different from investigations in soil ecology (e.g. process advancement in biotechnology with no substrate limitations vs. questions of C stabilization in resource scarce soils).

(ii) The absence of non-growth anabolism from CUE calculations has been justified by its presumably low contribution to overall anabolism under aerobic conditions (Manzoni et al., 2018; Paul and Clark, 1989). This assumption can, however, be questioned, because it is based on glucose tracer experiments (Frey et al., 2001; Šantrůčková et al., 2004) in which glucose was applied in quantities well above the range commonly found in soils (i.e. 315-1000 µg glucose-C g⁻¹ soil vs. 0.012-216 µg glucose-C g⁻¹ soil, respectively; Dijkstra et al., 2015; Frey et al., 2001; Šantrůčková et al., 2004). Previously, high rates of glucose addition have been criticised for distorting insights into microbial metabolism and CUE, because glucose can trigger rapid uptake and intracellular storage and/or favour rapid growth of r-selective microorganisms over more versatile metabolic performance (Blagodatskaya et al., 2014; Dijkstra et al., 2015; Sinsabaugh et al., 2013). Neglecting non-growth anabolism for CUE investigations is thus only justified from experiments favouring growth over non-growth anabolism, investigating CUE under uncommon soil conditions. The criticism of high glucose application rates has led to experiments reflecting more realistic, in-situ conditions (e.g. Bölscher et al., 2017; Dijkstra et al., 2015; Jones et al., 2019; Takriti et al., 2018), and promoting the development of methods independent of ¹³C- or ¹⁴C-labelled substrate addition (Blazewicz and Schwartz, 2011; Canarini et al., 2020; Spohn et al., 2016). Yet surprisingly, it did not trigger a critical re-consideration regarding the neglect of non-growth anabolism for CUE.
(iii) Although quantifying microbial non-growth anabolism and its metabolites in soils remains challenging, advancements have been made and deserve attention (Banfield et al., 2017; Mason-Jones et al., 2023, 2019; Redmile-Gordon et al., 2015, 2014). In the following, we will demonstrate the need to consider non-growth anabolism for CUE. We will then discuss how CUE investigations can be advanced using readily available methods that quantify at least some products of non-growth anabolism.

3. Advancing the concept and calculation of CUE in soil

Neglecting of non-growth anabolism is not only affecting the concept of CUE (i.e. viewing that non-growth C is ‘inefficiently’ used for synthesis of waste products) but also impacts how CUE in soil is quantified (Fig. 1). Carbon used for non-growth anabolism is virtually absent from common CUE measurements. Approaches either trace (i) $^{13}$C or $^{14}$C from labelled substrate into microbial biomass and CO$_2$-respiration (i.e. substrate-specific CUE; Frey et al., 2001; Geyer et al., 2019; Manzoni et al., 2012) or (ii) $^{18}$O from labelled water into microbial DNA. The latter is then used to estimate growth in combination with biomass measurements and complimented with non-isotope-specific measurements of CO$_2$-respiration (i.e. substrate-independent CUE; Blazewicz and Schwartz, 2011; Canarini et al., 2019; Geyer et al., 2019; Spohn et al., 2016). In both approaches, the total C metabolized by microorganisms is calculated as the sum of the C used for microbial respiration and growth, with the latter being estimated from DNA and/or biomass measurements (Geyer et al., 2019) (Fig. 1a):

$$\text{CUE}_{\text{apparent}} = \frac{C_{\text{growth}}}{C_{\text{growth}} + C_{\text{respiration}}}$$  

(1)

Where $\text{CUE}_{\text{apparent}}$ is the apparent CUE and $C_{\text{growth}}$ and $C_{\text{respiration}}$ are C used for growth or respiration, respectively. Here we propose that equation (1) provides only an apparent CUE
because it does not consider the entire metabolized C by microorganisms as non-growth anabolism is absent from the calculation (Fig. 1a). It illustrates that microbial extracellular metabolites are not quantified for substrate-specific CUE and even all non-growth metabolites (i.e. extra- and intracellular) are not captured when using substrate-independent CUE. For investigating SOC stabilization, we propose that actual CUE should be calculated, considering growth and non-growth anabolism (Fig 1b):

\[
CUE_{\text{actual}} = \frac{C_{\text{growth}} + C_{\text{non-growth}}}{C_{\text{growth}} + C_{\text{non-growth}} + C_{\text{respiration}}} \tag{2}
\]

Where \(CUE_{\text{actual}}\) is the actual CUE and \(C_{\text{non-growth}}\) is C used in non-growth anabolism (Fig. 1b). From equation (2), it becomes clear that not capturing non-growth anabolism could lead to an underestimation of CUE because \(C_{\text{non-growth}}\) appears in the numerator and denominator of the equation. In the following, we will quantify potential underestimation of CUE when non-growth metabolites are excluded using data from two published studies two examples (i.e. extracellular enzymes, Domeignoz-Horta et al., 2023, and EPS, Olagoke et al., 2022).

--- approximate position Figure 1 ---

4. Accounting for microbial non-growth anabolism reveals underestimations of actual CUE.

We employed two approaches to quantify potential underestimation of \(CUE_{\text{actual}}\) when non-growth anabolism is not quantified during CUE measurements (Domeignoz-Horta et al., 2023, Olagoke et al., 2022; section 4.1 and 4.2, respectively). Then, we performed a theoretical exercise assuming various relative allocations of C during metabolism towards non-growth versus growth anabolism (section 4.3). In all cases, \(CUE_{\text{actual}}\) versus \(CUE_{\text{apparent}}\) was expressed as a unitless number between 0.00 and 1.00 (i.e. 0-100% efficiency).
4.1. Case study 1: What is the effect of extracellular enzyme production on CUE?

We used data on extracellular enzymes and substrate-independent CUE, measured by Domeignoz-Horta et al. (2023), to compare CUE_{actual} and CUE_{apparent} (for detailed information, see Supplementary Methods): In a first scenario (enzyme pool maintenance), we assumed that the microbial community invests C only into non-growth anabolism in order to maintain the existing pool of exoenzymes. In a second scenario (enzyme pool expansion), we assume that the microbial community expands the exoenzyme pool by 20%. Similar enzyme pool increases were observed previously following shifts in soil nutrient inputs (Allison and Vitousek, 2005).

Depending on the underlying scenario (Fig. 2, Table S1), our analysis demonstrates that exoenzyme production can influence CUE to contrasting degrees. For the first scenario, enzyme pool maintenance, the underestimation of CUE_{actual} is minute, i.e. less than 0.01 differences between CUE_{actual} and CUE_{apparent} (Fig. 2a and b). This result indicates that microbial investments of C into exoenzymes may remain negligible for CUE measurements when merely compensating for enzyme turnover. But, for the enzyme pool expansion scenario, we found substantial underestimation of CUE_{actual}. The assumed 20% increase in the exoenzyme pool caused underestimations of CUE_{actual} between 0.002 and 0.189 (Fig. 2c and d). Here, 40% of the samples would show an underestimation of 0.05 or larger and in 12% of the samples CUE_{actual} would be more than twice as high as CUE_{apparent}. Consequently, even smaller increases than the assumed 20% in the exoenzyme pool (e.g. 5% or 10% increase) could cause considerable underestimation of CUE_{actual}. These findings demonstrate that microorganisms can potentially invest an important fraction of their C budget into the production of enzymes. We therefore consider that microbial C investments into enzymes should not be—a priori—ignored during investigations of CUE.
The high sensitivity of $\text{CUE}_{\text{actual}}$ to increased enzyme production calls for a better understanding of enzyme pool dynamics in soils and their effects on CUE. Generally, it can be assumed that the formation and turnover of enzymes, thus the size of the exoenzyme pool, is dynamic in soils (Schimel et al., 2017; Sinsabaugh, 2010; Zuccarini et al., 2023). Both, enzyme production and turnover depend on the environmental conditions, such as organic matter quality, nutrient availability, season, or soil moisture (Allison and Vitousek, 2005; Zuccarini et al., 2023) and change considerably over short time (Allison and Vitousek, 2005). To advance our knowledge whether neglecting exoenzymes affects CUE, we call for measuring exoenzyme pool dynamics over time and in relation to microbial growth. We need to further our understanding of (environmental) conditions when microorganisms (i) merely maintain existing enzyme pools and invest little C into new exoenzyme, and (ii) when they increase their C investment to expand the exoenzyme pools. Especially, it is necessary to take further consideration in relation to growth dynamics, because increased exoenzyme formation is generally followed by accelerated microbial growth (Schimel and Weintraub, 2003). It remains, however, unclear in how far these coupled—yet shifted in time—changes affect the underestimation of $\text{CUE}_{\text{actual}}$ over time. Yet, due to the time-shift, it can be assumed that $\text{CUE}_{\text{actual}}$ remains high over longer periods than $\text{CUE}_{\text{apparent}}$, because $\text{CUE}_{\text{apparent}}$ is only affected by accelerated growth while $\text{CUE}_{\text{actual}}$ increases already with the preceding increase in enzyme formation.

In the second scenario, exoenzyme pool expansion, the underestimation of $\text{CUE}_{\text{actual}}$ was dependent on measured $\text{CUE}_{\text{apparent}}$. We found a clear trend that the underestimation of $\text{CUE}_{\text{actual}}$ decreased with increasing $\text{CUE}_{\text{apparent}}$ (Fig. 2d). This trend occurs, because the relative distribution of C between growth and non-growth anabolism is of significance for CUE.
Assuming a fixed amount of C allocated to enzyme production will have a stronger effect on
CUE_{actual} when less C is used for growth compared with a scenario when much C is used for
growth. Substrate-independent methods (i.e. $^{18}$O tracing techniques) commonly measure
CUE_{apparent} for SOM decomposition less than 0.40 (Geyer et al., 2019). As exoenzyme-related
underestimations of CUE_{actual} seem to be larger below this value, special considerations should
be given to potential effects of exoenzyme pool dynamics on CUE.

4.2. Case study 2: What is the effect of EPS production on CUE?

We used data from a soil microcosms experiment by Olagoke et al. (2022) and compared
hypothetical CUE_{apparent} with CUE_{actual} when EPS production was quantified (for detailed
information, see Supplementary Methods). For our purpose, we focused on two clay treatments
(i.e. +0% and 1+% clay) in combination with three substrate additions (i.e. starch, cellulose and
no substrate). Since no CUE was measured in the experiment, we assumed that CUE_{apparent} can
range between 0.10 to 0.80 in each sample. We then calculated the respective cumulative
respiration and CUE_{actual} based on the measured changes in EPS and microbial biomass C as well
as the assumed CUE_{apparent} (see Supplementary Methods). Based on real EPS and microbial
biomass C data, this approach provided us with a set of hypothetical CUE_{apparent} and CUE_{actual} for
each treatment.

Our analysis revealed that the underestimation of CUE_{actual} can be considerable when microbial
EPS production is neglected (Fig. 3, Supplementary Table S2 and S3). Depending on the amount
of produced EPS relative to microbial biomass, the underestimation of CUE_{actual} can span from
virtually 0 (see 0% clay and starch addition treatment, assuming CUE_{apparent} of 0.10; Fig 3b) up
to 0.12 (1% clay and cellulose addition treatment, assuming CUE_{apparent} of 0.40 and 0.50; Fig 3b).
In the latter case, CUE_{actual} would be 0.52 or 0.62, respectively (Fig. 3a). The implication of these
underestimations becomes clearer when we consider the relative differences in the two approaches to calculate CUE. In our case, CUE\textsubscript{actual} would be 4 to 30\% higher than CUE\textsubscript{apparent}, which means that up to 30\% more C may potentially be stabilized than estimated in the approach neglecting microbial non-growth anabolism.

Furthermore, in all treatments, underestimations of CUE\textsubscript{actual} peak between 0.40 to 0.50 CUE\textsubscript{apparent} and decreases towards both ends of the CUE range (i.e. 0.10 to 0.80, Fig. 3b). Thus, CUE\textsubscript{actual} could be substantially underestimated over the range of usually reported CUEs in soils (Dijkstra et al., 2015; Geyer et al., 2019; Sinsabaugh et al., 2013), the range of CUE usually assumed in soil C models (Parton et al., 1987; Manzoni et al., 2012). Studies using labelled glucose have been criticized for triggering rapid growth and uncommonly high CUE (Geyer et al., 2019; Sinsabaugh et al., 2013). Some soil C models consider therefore lower CUE (e.g. the Q-model assuming CUE of ~0.25; Ågren and Bosatta, 1987) or substrate-independent methods are adopted (i.e. \textsuperscript{18}O tracer techniques; Blazewicz and Schwartz, 2011; Canarini et al., 2020; Spohn et al., 2016) resulting in CUEs below 0.40 (Geyer et al., 2019). Yet, even in this lower range, underestimations of CUE\textsubscript{actual} can remain high and may reach as much as 0.12 (Fig. 3b).

Underestimations of CUE\textsubscript{actual} seem to depend on the metabolized substrate. While we found relatively small underestimations of CUE\textsubscript{actual} for soil amended with starch (i.e. less than 0.02; Fig. 3b), the underestimations were considerably higher for soils amended with cellulose or unamended soils (i.e. 0.03-0.12 and 0.03-0.08, respectively, respectively; Fig. 3b). Differences were related to the relative production of EPS to biomass (Fig. 3c; Olagoke et al., 2022). Starch is readily available substrate for microbial metabolization, while cellulose breakdown is more complex and control soil were depleted of labile and particulate SOC (Olagoke et al., 2022).
Assuming a general bias in substrate-dependent underestimation of CUEactual has two consequences for future CUE investigations:

First, underestimations of CUEactual could be less pronounced (i) in the rhizosphere where roots exude low-molecular-weight organic compounds, (ii) at locations receiving fresh dissolved organic C via leaching, or (iii) at declining hot-spots of microbial activity with increased cell lysis (Kuzyakov and Blagodatskaya, 2015). Yet, underestimations of CUEactual could be more pronounced if organic C supply for microorganisms is low and/or dominated by complex organic matter. In the latter case, underestimations of CUEactual may not only be caused by C investments into EPS production (analysed here in case study 2), but also by a need to produce exoenzymes (see case study 1, section 4.1).

The second consequence for CUE investigations is related to methodology. If the substrate-specific CUE method is applied, addition of readily available substrates (e.g. glucose) could lead to lower underestimations of CUEactual than using more complex substrates (e.g. lignin or phenol; Bahri et al., 2008; Bölscher et al., 2017; Frey et al., 2013) or applying the substrate-independent method (i.e. the ¹⁸O-techniques; Blazewicz and Schwartz, 2011; Canarini et al., 2020; Spohn et al., 2016) which measures CUE on native complex SOC.

4.3. Theoretical approach: What is the effect of the relative distribution between non-growth and growth anabolism on CUE?

Our aim here was to evaluate the quantity of non-growth metabolites required to cause substantial underestimation of CUEactual. Because quantitative information on non-growth metabolites in soils is limited, we considered a theoretical approach using various relative microbial C allocation ratios between non-growth and growth anabolism (see Supplementary Methods). In general, underestimations of CUEactual increase with increasing amounts of C used...
for non-growth anabolism relative to growth (Fig. 4; Table S4). They become larger than 0.05
when the C used for non-growth anabolism is 50% or more than the C used for growth. The
graphs showing the underestimation of CUE\textsubscript{actual} follow inverse U-shapes peaking around a
CUE\textsubscript{apparent} of 0.35-0.50. At low CUE\textsubscript{apparent}, most of the total C used is directed towards
respiration and C\textsubscript{respiration} dominates the CUE calculations (equations (1) and (2)). Adding C\textsubscript{non-growth} to the calculation has therefore relatively small effect on CUE. At high CUE\textsubscript{apparent} most of
the C is used for growth. As C\textsubscript{growth} dominates the CUE calculation, adding C\textsubscript{non-growth} to the
calculation has also a realistic small effect. Considering C\textsubscript{non-growth} for CUE has the largest effect
on CUE values when the distribution of C between respiration, growth and non-growth
anabolism is balanced, a range of CUE\textsubscript{apparent} close to commonly measured in soils and
considered in soil C models (Dijkstra et al., 2015; Geyer et al., 2019; Parton et al., 1987;
Sinsabaugh et al., 2013). Our theoretical approach demonstrates that non-growth anabolism
should not be neglected for CUE in situations when non-growth C is equal or more than 50%
growth C and when CUE is expected to be in the midium range of potential CUES.

--- approximate position Figure 4 ---

5. Modelling approach: Potential consequences for SOC projections

To test how sensitive SOC projections are to varying CUEs, we applied a model introduced by
Meurer et al. (2020). Here, we introduced step-wise increase in the CUE model parameter
covering the underestimations of CUE\textsubscript{actual} found in the case studies and theoretical approach (see
Supplementary Methods).

In the model reference scenario (i.e. CUE = 0.14, assuming no underestimation of CUE\textsubscript{actual}),
SOC stocks are modelled with 4.21 kg C m\textsuperscript{2}. Yet, for the largest difference in CUE (i.e.
underestimation of CUE\textsubscript{actual} being 0.23, non-growth anabolic C equal to 150% of growth C, see
red solid line in Fig. 4), SOC stocks are modelled with 5.97 kg C m$^{-2}$, a SOC stock which is 42% larger than the reference scenario (Fig. 5). Also, we found almost as large discrepancies in modelled SOC stocks when the CUEs from the empirical case studies were applied. In the exoenzyme case, differences in CUE$_{actual}$ and CUE$_{apparent}$ ranged from no differences to 0.19 (Fig. 2) and calculated SOC stocks range from 4.21 kg C m$^{-2}$ (i.e. the reference value) to 5.63 kg C m$^{-2}$. The latter is 34% higher than the reference scenario (Fig. 5). In the EPS case study, CUE$_{actual}$ was 0.004 to 0.12 units higher than CUE$_{apparent}$ (Fig. 3). Modelled SOC stocks range from 4.22 to 5.10 kg C m$^{-2}$. While the former resulted in only a small discrepancy of 0.2% to the reference scenario, the latter is 21% larger than the reference scenario (Fig. 5b).

--- approximate position Figure 5 ---

Our modelling approach shows how crucial accurate estimations of CUE are for SOC projections, because an underestimation of CUE as little as 0.03 caused 5% higher SOC stocks, and that non-growth anabolism should not be disregarded from CUE measurements.

6. Perspective on microbial CUE in the light of SOC stabilization

6.1. Non-growth anabolism consumes likely a major part of microbially processed C

Quantifying microbial non-growth anabolism in soils remains challenging because the complex nature of the soil matrix hampers extraction, separation and analysis of non-growth metabolites. Yet, we show here that non-growth anabolites can make up a substantial part of microbially processed C and thus affect microbial CUE. The findings of our case studies (section 4) are supported by other (semi-)quantitative investigations. First of all, non-growth conditions are expected to dominate in soils, where access to available substrate and nutrients is restricted (Hobbie and Hobbie, 2013; Joergensen and Wichern, 2018; Kuzyakov and Blagodatskaya,
While the non-growth state of microorganisms remains largely uncharacterized in respect to metabolite production, recent studies suggest that metabolic activity and production can be substantial without microbial growth (Chodkowski and Shade, 2020; Joergensen and Wichern, 2018; Lever et al., 2015). Furthermore, it is widely accepted that the vast majority of bacteria and archaea in soils, as well as certain fungi, are surrounded by an EPS matrix (Costerton et al., 1987; De Beeck et al., 2021; Flemming and Wingender, 2010; Flemming and Wuertz, 2019). This matrix consists of polysaccharides, proteins, lipids and nucleic acids which account for 90% of the EPS matrix, while microbial cells account for less than 10% of its dry mass (Flemming and Wingender, 2010). Chenu (1995) estimated that microbial EPS in soil could be quantitatively equal to microbial biomass, representing up to 1.5% of SOC. Microbial cellular storage compounds are another form of non-growth C which is not accounted for by common organic C and DNA extractions. Mason-Jones et al. (2023) demonstrated recently that storage compounds could be of similar quantity as microbial biomass, even under C-limited conditions, counting for up to 19-46% of the extractable microbial biomass C and a biomass increase as large as 2.8 fold accounted for by DNA-based techniques. Other examples of non-growth anabolites are osmolytes, which can account for 10% or more of microbial biomass (Schimel et al., 2007; Warren, 2020), and oxalic acid, which was released by mineral weathering fungi in quantities equal to 1-20% of the fungi’s biomass during a 19 hours incubation experiment (Schmalenberger et al., 2015). These examples represent a glimpse of studies to illustrate that soil microbial communities produce a diverse set of non-growth metabolites, potentially in quantities which can be crucial when estimating CUE values. Yet, the quantities of non-growth metabolites produced in-situ remain uncertain. Non-growth metabolites should thus not be forgotten, especially as microbial communities likely synthesise various non-growth metabolites.
simultaneously. Further attention should be given to improving techniques to quantify non-
growth metabolites in soil.

6.2. Non-growth metabolites in soils are likely as stable as residues of microbial growth

Microbial non-growth metabolites may not only be quantitatively important for CUE, but may
also be a stable soil C pool. It is suggested that residues of microbial growth make up a major
part of SOC (Deng and Liang, 2021; Kallenbach et al., 2015; Liang et al., 2017; Miltner et al.,
2012). Although less studied, microbial non-growth metabolites in soils may be as stable as
microbial growth residues and foster processes that promote C stabilization, such as soil
aggregation and formation of mineral-organic associations (Chenu and Stotzky, 2002; Kleber et
al., 2015). Non-growth metabolites, like EPS or exoenzymes, are built of proteins,
polysaccharides, lipids, and other polymeric substances (Burns et al., 2012; Flemming and
Wingender, 2010). These substances have a high affinity to reactive mineral surfaces. They form
strong multiple bonds, due to a diverse set of molecular functional groups, via various
mechanisms (Kleber et al., 2021, 2015, 2007; Lehmann et al., 2020), promoting the formation of
relatively stable forms of mineral-organic associations (Chenu, 1995; Chenu and Stotzky, 2002;
Yang et al., 2021). Additionally, it is widely established that EPS contribute to SOC stabilization
via soil aggregation (Guhra et al., 2022). These examples demonstrate that non-growth
anabolism does not only serves important functions for microbial survival, but likely contributes
to stable SOC in similar ways as residues of microbial growth. Recent findings indicate even
that products of non-growth anabolism may be more important for forming stable SOC than
products of growth (Craig et al., 2022).

6.3. We need to open the ‘black box’ of microbial physiology in soil
The concept of CUE is ambiguous (Schimel et al., 2022) and treats microbial physiology as a ‘black box’. While its ambiguity has received attention (e.g. Geyer et al., 2019, 2016; Joergensen and Wichern, 2018; Manzoni et al., 2018; Schimel et al., 2022), its ‘black box’ character has been rarely considered (Dijkstra et al., 2022). Here, we argue for a need to open the ‘black box’ of CUE, supporting Dijkstra et al. (2022) in their call to disentangle the underlying metabolic processes, including those of non-growth anabolism. Efforts in this direction seem more promising to advance our knowledge than attempts to overcome the CUE ambiguity via additional efficiency definitions (e.g. Cai et al., 2022; Geyer et al., 2016; Manzoni et al., 2018). Strictly speaking, the latter provides primarily additional ‘black boxes’ of varying sizes rather than furthering our process understanding. To advance our understanding of the microbial physiology’s role in SOC stabilization, we need to disentangle the different pathways of microbial anabolism, improve our abilities to quantify the various products of microbial physiology (i.e. endogenous and exogenous) in soil and understand the environmental influence on their dynamics.

How do habitat conditions in combination with microbial life-history strategies influence the microbial metabolic C allocation? It can be assumed that most non-growth metabolites (such as EPS, enzymes and osmolytes) show linked dynamic behaviours, following environmental changes within the microbial habitat (Redmile-Gordon et al., 2015; Schimel et al., 2007; Zuccarini et al., 2023). For example, fresh inputs of complex organic C can trigger an increase in production of exoenzymes (Allison and Vitousek, 2005), followed by a delay in microbial growth (Schimel and Weintraub, 2003), which then is followed by an increase in formation of EPS when substrate becomes scares (Olagoke et al., 2022). Such associated dynamics of metabolite production have consequences for CUE measurements. In our example, $CUE_{\text{apparent}}$
would only increase for a short time with enhanced microbial growth, while $\text{CUE}_{\text{actual}}$ would remain on a high level for an extended period from the start of enzyme production until EPS formation subsides. To advance our understanding of CUE and metabolite dynamics, we need to establish procedures with repeated measurements of CUE and microbial metabolites over short periods, but also over seasonal shifts throughout the year.

The research aims laid out here require that we develop new and advance existing techniques for identification of metabolic C fluxes and quantify endo- and exogenous metabolites in soil. To date, advances have been made to quantify, for instance, microbial EPS (Redmile-Gordon et al., 2015, 2014), storage compounds (Banfield et al., 2017; Mason-Jones et al., 2019) and extracellular enzyme C (this study, see Supplementary Methods). $^{13}$C Metabolic Flux Analysis offers a way forward to track C fluxes during metabolism. It measures active metabolic pathways via the incorporation of $^{13}$C from position-specific labelled substrate into products of biosynthesis (Zamboni et al., 2009) or CO$_2$ (Dijkstra et al., 2022, 2015, 2011). $^{13}$C Metabolic Flux Analysis may offer great potential for opening the black boxes of soil microbial physiology and CUE, especially in combination with improved extraction procedures, metabolomic approaches and other “omics” technologies (e.g. transcriptomics and genomics; Chowdhury et al., 2021; Daniel, 2005).

Capturing entire microbial metabolic C fluxes in soil is currently impossible and it will likely remain a major challenge in the near future. Adopting the ‘black box’ of CUE is advantageous e.g. as a simple indicator for large-scale SOC projections (reducing required input data and computing capacity). But, we will need to understand the underlying processes of microbial physiology to judge when current simpler CUE measurements are sufficient (i.e. equation (1)) or when more inclusive complex CUE measurements are required (i.e. equation (2)). In the end, the
development around microbial CUE may go in parallel with the development of soil C models, where complex mechanics models provide process understanding while simple kinetic-based models are commonly used for large-scale SOC projections (Noë et al., 2023). While the complex mechanistic soil C models would profit from an ‘open box’ of soil microbial physiology, simple soil C models would profit from improved measurements of actual CUE, keeping this physiological feature as a ‘black box’.

7. Conclusions

Carbon used for non-growth anabolism is commonly disregarded in estimations of microbial CUE. Thus, CUE values represent only ‘apparent’ CUEs. In the light of SOC stabilization, non-growth anabolism is essential and needs to be quantified to capture entire microbial C use and measure ‘actual’ CUE. Here, we argue for an adjustment of microbial CUE measurements. Using two case studies and a theoretical approach, we demonstrated that measurements of apparent CUE can substantially underestimate actual CUE, especially over a CUE range commonly observed in soils. Considering an exoenzyme pool expansion by 20% will result in doubling of CUE values, while considering EPS production increased CUE by up to 30%. A SOC model reacted highly sensitive when we increased the CUE parameter similarly, projecting up to 34% larger SOC stocks after 64 years. Although quantification of non-growth metabolites in soils remains challenging, efforts should be made to further our understanding of their role in the terrestrial C cycle. Microbial communities may invest substantial amount of metabolized C into non-growth metabolites, which are likely as much stabilized in soils as residues of microbial growth. Both metabolite types are exposed to the same C stabilization mechanisms. We call for efforts to open the ‘black box’ of microbial physiology, represented by CUE, to advance our mechanistic understanding of how microbial physiology contributes to stabilized SOC. Recent
advances allow us to quantify, to some degree, non-growth metabolites such as EPS, exoenzymes and storage compounds. Efforts in this direction should continue and acknowledge the dynamic, linked nature of the various microbial C pathways and their dependence on conditions in the microbial habitat, an underexplored research area in terrestrial C cycle.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Fig. 1. Schematic illustration of carbon (C) partitioning during microbial metabolism when considering carbon-use efficiency (CUE). (a) Current concepts of CUE disregard C used for non-growth anabolism (Cnon-growth). CUE is quantified from C used for growth (Cgrowth) and respiration (Crespiration) where the entire C uptake (Cuptake) is considered as the sum of Cgrowth and Crespiration (equation (1)). Current approaches measure therefore an apparent CUE (CUEapparent). (b) To measure actual CUE (CUEactual), Cnon-growth should be considered. The latter remains in soil at the time and therefore needs to be included in the numerator and denominator of the CUE equation, if soil C stabilization is of interest (equation (2)).
Fig. 2. Actual and apparent carbon-use efficiencies (CUE\textsubscript{actual} and CUE\textsubscript{apparent}, respectively) considering extracellular enzyme formation based on data adopted from Domeignoz-Horta et al. (2023). The displayed results consider two scenarios for enzyme formation: First, microbial communities maintaining the existing exoenzyme pool by replacing turned-over exoenzymes (a, b). Second, an expansion of the exoenzyme pool by 20\% (c, d). Left panels (a, c) compare CUE\textsubscript{apparent} (equation (1)) and corresponding CUE\textsubscript{actual} (equation (2)) for the two scenarios, respectively. The dotted lines indicate the 1:1 ratio of equal CUE\textsubscript{apparent} and CUE\textsubscript{actual}. Right panels (b, d) present the underestimation of CUE\textsubscript{actual} (i.e. CUE\textsubscript{actual} minus CUE\textsubscript{apparent}) plotted as function of assumed CUE\textsubscript{apparent} for the two scenarios, respectively.
Fig. 3. Actual and apparent carbon-use efficiencies (CUE$_{\text{actual}}$ and CUE$_{\text{apparent}}$, respectively) and production of extracellular polymeric substances (EPS) calculated from data of Olagoke et al. (2022). (a) Comparison between assumed CUE$_{\text{apparent}}$ (equation (1)) and corresponding CUE$_{\text{actual}}$ (equation (2)) for soil treated with cellulose, starch or no substrate (i.e. control) in combination with either +0 or +1% clay. The dotted line indicates the 1:1 ratio of equal CUE$_{\text{apparent}}$ and CUE$_{\text{actual}}$. (b) Underestimation of CUE$_{\text{actual}}$ (i.e. CUE$_{\text{actual}}$ minus CUE$_{\text{apparent}}$) plotted as a function of assumed CUE$_{\text{apparent}}$. (c) Production of EPS carbon (EPS-C) relative to the change in microbial biomass carbon (ΔMBC) after substrate addition. Results are displayed as means and error bars show standard errors (n = 4). If no whiskers are visible, standard errors are smaller than the symbol size. Symbols in (a) and (b) are slightly shifted along the x-axis to improve visibility.
Fig. 4. Underestimation of actual CUE ($\text{CUE}_{\text{actual}}$) in relation to microbial carbon (C) allocation between non-growth and growth anabolism. Underestimation of $\text{CUE}_{\text{actual}}$ (i.e. $\text{CUE}_{\text{actual}}$ minus $\text{CUE}_{\text{apparent}}$) is plotted as function of assumed apparent CUE ($\text{CUE}_{\text{apparent}}$). $\text{CUE}_{\text{apparent}}$ was assumed to range between 0.10 and 0.80. $\text{CUE}_{\text{actual}}$ was calculated for seven scenarios with C used for non-growth anabolism relative to C used for growth ranging from 5 to 150% of C used for growth.
Fig. 5. Results from the modelling approach showing soil organic carbon (SOC) stocks calculated for the Green Manure treatment of the Ultuna Long-Term Soil Organic Matter Experiment (Herrmann and Witter, 2008; Persson and Kirchmann, 1994) in the year 2020. The SOC stocks were modelled assuming CUEs in the range from 0.14 (i.e. the model reference value) to 0.37, a similar range as observed in the two case studies and theoretical approach (section 4). (a) On the left, calculated SOC stocks are shown, (b) while differences in SOC stocks to the reference (i.e. no underestimation of CUEactual) are shown on the right. Horizontal solid lines above the graphs show the range of the underestimation of CUEactual found in the case studies and theoretical approach. For the case study of extracellular polymeric substances (EPS) and the theoretical approach, markers are placed on the lines where the value is located for an assumed CUEapparent of 0.14 (i.e. the model reference CUE). In the EPS case, round markers are used for the +0% clay treatment and triangular markers for the +1% clay treatment. The dotted horizontal line in the graphs represents (a) the SOC stock or (b) difference to the reference SOC stock assuming an annual increase by 4 ‰ (Rumpel et al., 2020) until 2020, relative to the initial stocks in 1956.
Supplementary Materials for

Beyond growth: the significance of non-growth anabolism for microbial carbon-use efficiency in the light of soil carbon stabilisation

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Supplementary Methods.

Supplementary Note. Consequences of neglecting non-growth anabolism for investigating microbial growth efficiency.

Fig. S1. Comparison between actual and apparent microbial growth efficiency.

Fig. S2. Maximal formation of total extracellular polymeric substances (EPS) carbon (EPS-C) relative to maximal production of microbial biomass carbon (MBC) over the entire 80 days incubation.

Table S1. Microbial carbon use efficiencies (CUE) calculated from the data by Domeignoz-Horta et al. (2023) (case study 1).

Table S2. Microbial biomass carbon (MBC) and extracellular polymeric substances (EPS) formation (case study 2).

Table S3. Microbial carbon use efficiencies (CUE) calculated from the data by Olagoke et al. (2022) (case study2).

Table S4. Microbial carbon use efficiencies (CUE) calculated based on the relative allocation of carbon (C) between non-growth and growth metabolites (theoretical approach).

Supplementary References.
SUPPLEMENTARY METHODS

Case study 1: What is the effect of extracellular enzyme production on CUE?

Domeignoz-Horta et al. (2023) measured substrate-nonspecific carbon-use efficiency (CUE) and extracellular enzymes in soil samples from two soil warming experiments, running since 13 and 28 years (i.e. Soil Warming and Nitrogen Addition Study and Prospect Hill, respectively), at the Harvard Forest Long-Term Ecological Research sites in Petersham MA USA (42°30′30″N, 72°12′28″W). For a detailed description, we refer to the original study (Domeignoz-Horta et al., 2023). In short, soil was sampled from soil warming (ambient +5 °C) and control plots (ambient; n = 5, both) in July and October 2019. Soil was separated in organic and mineral soil by layer, sieved on site (2 mm) and stored at 15 °C. Activities of beta-glucosidase (cellulose-degrading enzyme) and N-acetylglucosaminidase (chitin- and peptidoglycan-degrading enzyme) were assayed at several temperatures between 4 and 30 °C within four days of sampling (Domeignoz-Horta et al., 2023). Here, we focused our analysis on CUE and enzyme data measured at 20 °C, representing a mid-temperature within the growing season’s temperature range (Domeignoz-Horta et al., 2023). To quantify the carbon (C) investments associated to extracellular enzymatic activity, we divided the C allocation into investments associated to maintenance and expansion of the enzymatic pool. To estimate the C investment related to maintenance of the enzymatic pool, we considered first the enzyme turnover rates to be 0.004 d⁻¹ for beta-glucosidase and 0.0085 d⁻¹ and N-acetylglucosaminidase (Schimel et al., 2017). We then estimated the mean protein sizes to be made of 700 and 500 amino-acids for beta-glucosidase and N-acetylglucosaminidase, respectively, based on the NCBI protein database (NCBI, 2023). Finally, we considered the average C fraction by amino-acid in the proteins for each enzyme. To estimate the C investments related to the expansion of the enzymatic pool, we considered an expansion of the enzymatic pool by 20%. Comparable enzyme pool expansions have been found previously following shifts in soil nutrient inputs (Allison and Vitousek, 2005). Substrate-nonspecific CUE (i.e. CUE_{apparent}, ¹⁸O-water technique) was measured by Domeignoz-Horta et al. (2023) two days after sampling using the method described by Spohn et al. (2016).

We used the CUE values measured by Domeignoz-Horta et al. (2023) as CUE_{apparent} (equation (1)) and calculated CUE_{actual} (equation (2); see section 3 in main paper), assuming that the measured exoenzymes represented C_{non-growth} (i.e. we neglected all other non-growth metabolites here, but acknowledge that they may be quantitatively important).
Case study 2: What is the effect of EPS production on CUE?

Olagoke et al. (2022) investigated EPS production and changes in microbial biomass C (MBC) over time. For a detailed description, we refer to the original study (Olagoke et al., 2022). Briefly, a sandy soil received clay addition using montmorillonite after the removal of labile and particulate organic carbon. For our purpose, we concentrated on the +0% and +1% clay treatments (i.e. 4.4% and 5.4% final clay content). Soil microcosms were incubated at 20 °C over a period of 80 days after receiving either cellulose, starch or no substrate (i.e. rather ‘recalcitrant’, readily available or no C, respectively) to stimulate microbial activity. Microcosms were harvested at several time points during the incubation (including 0 and 3 days) and analysed for EPS-proteins, EPS-polysaccharides (Frølund et al., 1996; Olagoke et al., 2022; Redmile-Gordon et al., 2014) and MBC (Olagoke et al., 2022; Vance et al., 1987; Wu et al., 1990).

Here, we focus on changes in EPS and MBC between days 0 and 3 to calculate CUE\textsuperscript{apparent} and CUE\textsuperscript{actual} (equation (1) and (2), respectively). Focusing on the first three days of incubation reduced the risk of confounding CUE values due to recycling of microbial metabolites (Hagerty et al., 2014). Increases in MBC were considered as growth (i.e. C\textsubscript{growth}), while increases in EPS were considered as C used non-growth anabolism (i.e. C\textsubscript{non-growth}). Total EPS production was calculated by adding increases in EPS-proteins and EPS-polysaccharides. In one case (i.e. cellulose, +0% clay) where EPS-polysaccharides decreased, we assumed their production as zero. We derived the C used for respiration (i.e. C\textsubscript{respiration}) from changes in MBC (i.e. C\textsubscript{growth}), as cumulative respiration was not measured (Olagoke et al., 2022). Therefore, we used equation (1) and assumed a set of specific CUE\textsuperscript{apparent} values between 0.10 and 0.80. In other words, we solved equation (1), knowing C\textsubscript{growth} from the MBC change and an assumed CUE value, to calculate C\textsubscript{respiration}. For example, if C\textsubscript{growth} was 10 µg C g\textsuperscript{-1} soil and we assumed CUE\textsuperscript{apparent} to be ether 0.10, 0.50 or 0.80, we derived C\textsubscript{respiration} to be 90, 10 and 2.5 µg C g\textsuperscript{-1} soil, respectively (i.e. the total microbial C uptake—numerator in equation (1)—would be 100, 20, and 12.5 µg C g\textsuperscript{-1} soil, respectively). The highest assumed CUE value of 0.80 represents the approximate theoretical upper maximum of CUE (Hagerty et al., 2014).
Theoretical approach: What is the effect of the relative distribution of C between non-growth and growth anabolism on CUE?

For the theoretical approach, we first set the total amount of C metabolized to 100 units of C. We then calculate CUE\textsubscript{apparent} (equation (1)), assuming that the 100 units of C are allocated between respiration (i.e. $C_{\text{respiration}}$) and growth (i.e. $C_{\text{growth}}$) and that no C is used for non-growth anabolism. Here, we assumed that CUE\textsubscript{apparent} varies between 0.10 and 0.80, similar to the approach used in case study 2. Based on the assumed CUE\textsubscript{apparent}, the 100 units of C would be divided accordingly between $C_{\text{respiration}}$ and $C_{\text{growth}}$. For example, if CUE\textsubscript{apparent} was set to 0.30 or 0.80, $C_{\text{growth}}$ would be 30 or 80 units of C, respectively. Accordingly, $C_{\text{respiration}}$ would be 70 or 20 units of C, respectively. We then assumed that a certain proportion of C is used to synthesize non-growth metabolites (i.e. $C_{\text{non-growth}}$) relative to the amount of $C_{\text{growth}}$. For instance, we would assume that $C_{\text{non-growth}}$ would be equal to 10% of $C_{\text{growth}}$. This means, that $C_{\text{non-growth}}$ would then be 3 or 8 units of C, respectively, in our previous example. Here, the total amount of C remaining in soil would be 33 or 88 units of C (i.e. $C_{\text{growth}}$ plus $C_{\text{non-growth}}$) rather than 30 or 80 units of C (i.e. $C_{\text{growth}}$ alone). In a last step, we calculated CUE\textsubscript{actual} (equation (2)). For our example, the values would be 0.32 or 0.81, as the total amount of C metabolized (i.e. denominator of equation (2)) would be 103 or 108 units of C rather than 100 units of C initially assumed, neglecting non-growth anabolism.

We assumed quantities of C used for non-growth anabolism equal to 5 to 150% of C used for growth. These numbers include the range of maximum biomass and EPS formation measured in the experiment by Olagoke et al. (2022) (Fig. S2). We further assumed that in certain situations more C is used for non-growth anabolism than for growth, addressing the different life-history strategies of microorganisms in soil. Especially K-selective (or oligotroph) microorganisms may, under resource scarcity, rather invest C into non-growth-based survival strategies, such as biofilm formation, than using C for growth (Fierer et al., 2007; Flemming et al., 2016). We captured such a scenario by assuming $C_{\text{non-growth}}$ equals 150% of $C_{\text{growth}}$. Recently, it was found that storage compound formation—a commonly unaccounted type of non-growth anabolism—can be as large as almost 300% of $C_{\text{growth}}$ (Mason-Jones et al., 2023).
Modelling approach: Potential consequences for SOC projections

To test how sensitive SOC projections are to the differences in CUE found in our case studies, we applied the model introduced by Meurer et al. (2020) and used data from the Green Manure treatment of the Ultuna Frame Trial (Sweden, started in 1956; Herrmann and Witter, 2008; Persson and Kirchmann, 1994). For this treatment, the model fitted best with a CUE of 0.14. In the modelling approach, we step-wise increased the CUE parameter from 0.14 to 0.37 to cover the range of differences between CUE_{actual} and CUE_{apparent} that we found in the case studies and theoretical approach (i.e. underestimations of CUE_{actual} of up to 0.23). The model was used to calculate the changes in SOC and bulk density from 1956 (start of the field trial) to 2020 in the Green Manure treatment. The modelled SOC content and bulk density were then used to calculate SOC stocks to a depth of 20 cm (i.e. the sampling depth during yearly field campaigns at the site; Persson and Kirchmann, 1994) for the year 2020.
SUPPLEMENTARY NOTE

Consequences of neglecting non-growth anabolism for investigating microbial growth efficiency

Microbial growth efficiency (MGE) is often used synonymously for carbon use efficiency (CUE; Schimel et al., 2022; Sinsabaugh et al., 2013). Here, we call for a distinction of MGE and CUE in the light of soil carbon (C) stabilization. By distinguishing, CUE would assign all microbially metabolized C remaining in soil as efficiently used (i.e. all C used for biosynthesis), while MGE would only assign C used for growth as efficiently used. In both cases, neglecting non-growth anabolism during quantification of efficiency can lead to incorrect values of efficiency. The entire microbial C use is not captured, because non-growth is ignored. Measured efficiencies are thus apparent rather than actual efficiencies. While the neglect of non-growth anabolism causes underestimations of actual CUE (see main article), it leads to overestimations of actual MGE.

Commonly, MGE is calculated as (del Giorgio and Cole, 1998):

\[ MGE_{\text{apparent}} = \frac{C_{\text{growth}}}{C_{\text{growth}} + C_{\text{respiration}}} \]  \hspace{1cm} (S1)

Where \( MGE_{\text{apparent}} \) is the apparent MGE and \( C_{\text{growth}} \) and \( C_{\text{respiration}} \) are C used for growth and respired, respectively. To fully capture all metabolized C, C used for biosynthesis of non-growth anabolites needs to be added to the numerator of equation (S1). This provides us with the actual MGE (\( MGE_{\text{actual}} \)):

\[ MGE_{\text{actual}} = \frac{C_{\text{growth}}}{C_{\text{growth}} + C_{\text{non-growth}} + C_{\text{respiration}}} \]  \hspace{1cm} (S2)

Where, \( C_{\text{non-growth}} \) is the C used for non-growth anabolism.

Comparing equations (S1) and (S2) demonstrates that \( MGE_{\text{apparent}} \) tends to overestimate the actual MGE. The degree of overestimation will depend on the amount of C used for non-growth biosynthesis (i.e. \( C_{\text{non-growth}} \)) relative to the amount of C used for growth (i.e. \( C_{\text{growth}} \)). In Fig. S1, we plotted the difference between \( MGE_{\text{actual}} \) and \( MGE_{\text{apparent}} \), depending on \( MGE_{\text{apparent}} \) as well as the relative amount of C used for non-growth anabolism (similar to our approach used for CUE; see section 4.3 of main article and Supplementary Methods). The results show that overestimations
of MGE increase with higher MGE and relatively larger amounts of C used for non-growth anabolism (Fig. S1). The further demonstrate that overestimations of MGE can be quite substantial, especially when large amounts of non-growth metabolites are produced by soil microorganisms. In situations where microorganisms receive large quantities of readily decomposable substrate – such as glucose, using substrate-specific $^{13/14}$C tracer techniques –, microorganisms are assumed to produce very little non-growth metabolites (Blagodatskaya et al., 2014; Dijkstra et al., 2015). Here, overestimates of MGE remain relatively small ($\leq 0.03$ for non-growth C equalling 5% of growth-C) even at high MGE. But, when larger amounts of non-growth metabolites are produced, MGE can be considerably overestimated even in the range of MGE commonly measured using substrate-nonspecific approaches (i.e. MGE$_{\text{apparent}} < 0.40$, using $^{18}$O tracer techniques; Blazewicz and Schwartz, 2011; Canarini et al., 2020; Geyer et al., 2019; Spohn et al., 2016).
Fig. S1. Comparison between actual and apparent microbial growth efficiency (MGE_{actual} and MGE_{apparent}, respectively) depending on the amount carbon (C) used for non-growth to growth anabolism. (a) Direct comparison between MGE_{actual} and MGE_{apparent} demonstrating the overestimation of MGE_{actual}. The solid black line indicates the 1:1 ratio where MGE_{actual} equals MGE_{apparent}. (b) Differences between MGE_{actual} and MGE_{apparent} as function of assumed MGE_{apparent}. 
Fig. S2. Maximal production of extracellular polymeric substances (EPS) carbon (EPS-C) relative to maximal production of microbial biomass carbon (MBC) over the entire 80 days incubation. EPS-C and MBC were measured in soil amended with cellulose or starch or controls soils receiving no substrate. All soils were amended with +0, +0.1, +1 or +10% clay. Calculations based on data from Olagoke et al. (2022). See Supplementary Methods for details.
**SUPPLEMENTARY TABLES**

**Table S1.** Microbial carbon use efficiencies (CUE) calculated from the data by Domeignoz-Horta et al. (2023). Apparent CUE ($CUE_{\text{apparent}}$) were measured using the $^{18}$O-DNA technique and calculated from respiration and growth. Carbon (C) used for maintaining and expanding the enzyme pool was calculated as described in the Supplementary Methods. Actual CUE ($CUE_{\text{actual}}$) were calculated using equation (2) for the enzyme pool maintenance and enzyme pool expansion scenario, respectively (see section 3 and 4.1 of main article and Supplementary Methods). ID = sample ID, PH = Prospect Hill, SWaN = Soil Warming and Nitrogen Addition Study.

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<td>0.334</td>
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<td>0.209</td>
<td>0.308</td>
</tr>
<tr>
<td>26</td>
<td>PH</td>
<td>heated</td>
<td>Organic</td>
<td>July</td>
<td>1.844</td>
<td>0.216</td>
<td>0.003</td>
<td>0.097</td>
<td>0.105</td>
<td>0.106</td>
<td>0.145</td>
</tr>
<tr>
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<td>PH</td>
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<td>July</td>
<td>1.450</td>
<td>0.083</td>
<td>0.004</td>
<td>0.141</td>
<td>0.054</td>
<td>0.057</td>
<td>0.134</td>
</tr>
<tr>
<td>44</td>
<td>PH</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>1.750</td>
<td>1.126</td>
<td>0.004</td>
<td>0.131</td>
<td>0.392</td>
<td>0.392</td>
<td>0.418</td>
</tr>
<tr>
<td>51</td>
<td>PH</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>1.683</td>
<td>1.388</td>
<td>0.002</td>
<td>0.066</td>
<td>0.452</td>
<td>0.452</td>
<td>0.463</td>
</tr>
<tr>
<td>58</td>
<td>PH</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>1.522</td>
<td>0.483</td>
<td>0.003</td>
<td>0.119</td>
<td>0.241</td>
<td>0.242</td>
<td>0.283</td>
</tr>
<tr>
<td>60</td>
<td>PH</td>
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<td>Organic</td>
<td>October</td>
<td>1.113</td>
<td>2.047</td>
<td>0.002</td>
<td>0.061</td>
<td>0.648</td>
<td>0.648</td>
<td>0.654</td>
</tr>
<tr>
<td>73</td>
<td>PH</td>
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<td>Organic</td>
<td>October</td>
<td>2.743</td>
<td>2.435</td>
<td>0.004</td>
<td>0.129</td>
<td>0.470</td>
<td>0.471</td>
<td>0.483</td>
</tr>
<tr>
<td>9</td>
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<td>heated</td>
<td>Organic</td>
<td>July</td>
<td>1.421</td>
<td>0.524</td>
<td>0.008</td>
<td>0.276</td>
<td>0.269</td>
<td>0.272</td>
<td>0.360</td>
</tr>
<tr>
<td>12</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>July</td>
<td>1.139</td>
<td>0.225</td>
<td>0.005</td>
<td>0.153</td>
<td>0.165</td>
<td>0.168</td>
<td>0.249</td>
</tr>
<tr>
<td>30</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>July</td>
<td>1.097</td>
<td>0.082</td>
<td>0.006</td>
<td>0.187</td>
<td>0.070</td>
<td>0.074</td>
<td>0.197</td>
</tr>
<tr>
<td>32</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>July</td>
<td>1.528</td>
<td>1.155</td>
<td>0.005</td>
<td>0.161</td>
<td>0.431</td>
<td>0.432</td>
<td>0.463</td>
</tr>
<tr>
<td>35</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>July</td>
<td>1.525</td>
<td>0.217</td>
<td>0.009</td>
<td>0.314</td>
<td>0.125</td>
<td>0.129</td>
<td>0.258</td>
</tr>
<tr>
<td>49</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>1.925</td>
<td>1.774</td>
<td>0.003</td>
<td>0.116</td>
<td>0.480</td>
<td>0.480</td>
<td>0.495</td>
</tr>
<tr>
<td>56</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>1.672</td>
<td>1.308</td>
<td>0.004</td>
<td>0.148</td>
<td>0.439</td>
<td>0.440</td>
<td>0.465</td>
</tr>
<tr>
<td>64</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>1.347</td>
<td>2.221</td>
<td>0.003</td>
<td>0.110</td>
<td>0.623</td>
<td>0.623</td>
<td>0.634</td>
</tr>
<tr>
<td>77</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>0.642</td>
<td>0.683</td>
<td>0.008</td>
<td>0.272</td>
<td>0.515</td>
<td>0.518</td>
<td>0.598</td>
</tr>
<tr>
<td>80</td>
<td>SWaN</td>
<td>heated</td>
<td>Organic</td>
<td>October</td>
<td>5.544</td>
<td>1.087</td>
<td>0.014</td>
<td>0.478</td>
<td>0.164</td>
<td>0.166</td>
<td>0.220</td>
</tr>
</tbody>
</table>
Table S2. Microbial biomass carbon (MBC) and extracellular polymeric substances (EPS) formation over the first three days of the incubation experiment by Olagoke et al. (2022). ΔMBC is change microbial biomass carbon. ΔEPS-C\textsubscript{total}, ΔEPS-C\textsubscript{polysacc}, and ΔEPS-C\textsubscript{proteins} is change in total, polysaccharide and protein EPS carbon, respectively. ΔEPS-C\textsubscript{total} is expressed as concentration per gram soil (second column) as well as relative to change in MBC (last column). E and MBC were measured in soil amended with cellulose or starch or controls soils receiving no substrate. All soils were amended with +0 or +1% clay. Values are means (n = 4) ± one standard error.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔMBC (µg g\textsuperscript{-1} soil)</th>
<th>ΔEPS-C\textsubscript{total} (µg g\textsuperscript{-1} soil)</th>
<th>ΔEPS-C\textsubscript{polysacc.} (µg g\textsuperscript{-1} soil)</th>
<th>ΔEPS-C\textsubscript{proteins} (µg g\textsuperscript{-1} soil)</th>
<th>ΔEPS-C\textsubscript{total} (%) ΔMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, +0% clay</td>
<td>24.3 ±3.6</td>
<td>8.3 ±1.8</td>
<td>0.0 ±0.0</td>
<td>8.3 ±1.8</td>
<td>38 ±11</td>
</tr>
<tr>
<td>Control, +1% clay</td>
<td>39.2 ±2.2</td>
<td>14.7 ±2.0</td>
<td>0.3 ±0.3</td>
<td>14.4 ±2.2</td>
<td>38 ±7</td>
</tr>
<tr>
<td>Cellulose, +0% clay</td>
<td>71.5 ±3.1</td>
<td>26.0 ±3.9</td>
<td>5.7 ±3.3</td>
<td>20.2 ±1.4</td>
<td>37 ±7</td>
</tr>
<tr>
<td>Cellulose, +1% clay</td>
<td>75.7 ±6.0</td>
<td>47.5 ±1.2</td>
<td>24.5 ±2.0</td>
<td>23.0 ±1.6</td>
<td>64 ±5</td>
</tr>
<tr>
<td>Starch, +0% clay</td>
<td>864.2 ±15.4</td>
<td>33.3 ±1.5</td>
<td>14.8 ±0.8</td>
<td>18.5 ±0.9</td>
<td>4 ±0</td>
</tr>
<tr>
<td>Starch, +1% clay</td>
<td>758.4 ±15.7</td>
<td>60.4 ±2.7</td>
<td>28.3 ±0.7</td>
<td>32.4 ±2.5</td>
<td>8 ±1</td>
</tr>
</tbody>
</table>
Table S3. Microbial carbon use efficiencies (CUE) calculated from the data by Olagoke et al. (2022). Apparent CUEs (CUE\textsubscript{apparent}) are assumed and based on equation (1). Actual CUE (CUE\textsubscript{actual}) as calculated using equation (2) (see section 3 of main article and Supplementary Methods). Values are means (n = 4) ± one standard error.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CUE\textsubscript{apparent}</th>
<th>0.10 ±0.01</th>
<th>0.20 ±0.02</th>
<th>0.30 ±0.02</th>
<th>0.40 ±0.02</th>
<th>0.50 ±0.02</th>
<th>0.60 ±0.02</th>
<th>0.70 ±0.02</th>
<th>0.80 ±0.02</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, +0% clay</td>
<td>CUE\textsubscript{actual}</td>
<td>0.13 ±0.01</td>
<td>0.26 ±0.02</td>
<td>0.37 ±0.02</td>
<td>0.48 ±0.02</td>
<td>0.58 ±0.02</td>
<td>0.67 ±0.02</td>
<td>0.76 ±0.02</td>
<td>0.84 ±0.01</td>
</tr>
<tr>
<td></td>
<td>CUE\textsubscript{actual} minus CUE\textsubscript{apparent}</td>
<td>0.03 ±0.01</td>
<td>0.06 ±0.02</td>
<td>0.07 ±0.02</td>
<td>0.08 ±0.02</td>
<td>0.07 ±0.02</td>
<td>0.07 ±0.02</td>
<td>0.06 ±0.02</td>
<td>0.04 ±0.01</td>
</tr>
<tr>
<td>Control, +1% clay</td>
<td>CUE\textsubscript{actual}</td>
<td>0.13 ±0.01</td>
<td>0.26 ±0.01</td>
<td>0.37 ±0.01</td>
<td>0.48 ±0.01</td>
<td>0.58 ±0.01</td>
<td>0.67 ±0.01</td>
<td>0.76 ±0.01</td>
<td>0.85 ±0.01</td>
</tr>
<tr>
<td></td>
<td>CUE\textsubscript{actual} minus CUE\textsubscript{apparent}</td>
<td>0.03 ±0.01</td>
<td>0.06 ±0.01</td>
<td>0.07 ±0.01</td>
<td>0.08 ±0.01</td>
<td>0.08 ±0.01</td>
<td>0.07 ±0.01</td>
<td>0.06 ±0.01</td>
<td>0.05 ±0.01</td>
</tr>
<tr>
<td>Cellulose, +0% clay</td>
<td>CUE\textsubscript{actual}</td>
<td>0.13 ±0.01</td>
<td>0.25 ±0.01</td>
<td>0.37 ±0.01</td>
<td>0.48 ±0.01</td>
<td>0.58 ±0.01</td>
<td>0.67 ±0.01</td>
<td>0.76 ±0.01</td>
<td>0.84 ±0.01</td>
</tr>
<tr>
<td></td>
<td>CUE\textsubscript{actual} minus CUE\textsubscript{apparent}</td>
<td>0.03 ±0.01</td>
<td>0.05 ±0.01</td>
<td>0.07 ±0.01</td>
<td>0.08 ±0.01</td>
<td>0.08 ±0.01</td>
<td>0.07 ±0.01</td>
<td>0.06 ±0.01</td>
<td>0.04 ±0.01</td>
</tr>
<tr>
<td>Cellulose, +1% clay</td>
<td>CUE\textsubscript{actual}</td>
<td>0.15 ±0.00</td>
<td>0.29 ±0.01</td>
<td>0.41 ±0.01</td>
<td>0.52 ±0.01</td>
<td>0.62 ±0.01</td>
<td>0.71 ±0.01</td>
<td>0.79 ±0.00</td>
<td>0.87 ±0.00</td>
</tr>
<tr>
<td></td>
<td>CUE\textsubscript{actual} minus CUE\textsubscript{apparent}</td>
<td>0.05 ±0.00</td>
<td>0.09 ±0.01</td>
<td>0.11 ±0.01</td>
<td>0.12 ±0.01</td>
<td>0.12 ±0.01</td>
<td>0.11 ±0.01</td>
<td>0.09 ±0.00</td>
<td>0.07 ±0.00</td>
</tr>
<tr>
<td>Starch, +0% clay</td>
<td>CUE\textsubscript{actual}</td>
<td>0.10 ±0.00</td>
<td>0.21 ±0.00</td>
<td>0.31 ±0.00</td>
<td>0.41 ±0.00</td>
<td>0.51 ±0.00</td>
<td>0.61 ±0.00</td>
<td>0.71 ±0.00</td>
<td>0.81 ±0.00</td>
</tr>
<tr>
<td></td>
<td>CUE\textsubscript{actual} minus CUE\textsubscript{apparent}</td>
<td>0.00 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
</tr>
<tr>
<td>Starch, +1% clay</td>
<td>CUE\textsubscript{actual}</td>
<td>0.11 ±0.00</td>
<td>0.21 ±0.00</td>
<td>0.32 ±0.00</td>
<td>0.42 ±0.00</td>
<td>0.52 ±0.00</td>
<td>0.62 ±0.00</td>
<td>0.72 ±0.00</td>
<td>0.81 ±0.00</td>
</tr>
<tr>
<td></td>
<td>CUE\textsubscript{actual} minus CUE\textsubscript{apparent}</td>
<td>0.01 ±0.00</td>
<td>0.01 ±0.00</td>
<td>0.02 ±0.00</td>
<td>0.02 ±0.00</td>
<td>0.02 ±0.00</td>
<td>0.02 ±0.00</td>
<td>0.02 ±0.00</td>
<td>0.01 ±0.00</td>
</tr>
</tbody>
</table>
Table S4. Microbial carbon use efficiencies (CUE) calculated in the theoretical approach based on the relative allocation of carbon (C) between non-growth and growth anabolism. Apparent CUEs ($CUE_{\text{apparent}}$) are assumed and represent equation (2). Actual CUE ($CUE_{\text{actual}}$) as calculated using equation (2) (see section 3 of main article and Supplementary Methods).

<table>
<thead>
<tr>
<th>Non-growth C (% growth C)</th>
<th>$CUE_{\text{apparent}}$</th>
<th>0.10</th>
<th>0.20</th>
<th>0.30</th>
<th>0.40</th>
<th>0.50</th>
<th>0.60</th>
<th>0.70</th>
<th>0.80</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.104</td>
<td>0.208</td>
<td>0.310</td>
<td>0.412</td>
<td>0.512</td>
<td>0.612</td>
<td>0.710</td>
<td>0.808</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.004</td>
<td>0.008</td>
<td>0.010</td>
<td>0.012</td>
<td>0.012</td>
<td>0.012</td>
<td>0.010</td>
<td>0.008</td>
</tr>
<tr>
<td>10%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.109</td>
<td>0.216</td>
<td>0.320</td>
<td>0.423</td>
<td>0.524</td>
<td>0.623</td>
<td>0.720</td>
<td>0.815</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.009</td>
<td>0.016</td>
<td>0.020</td>
<td>0.023</td>
<td>0.024</td>
<td>0.023</td>
<td>0.020</td>
<td>0.015</td>
</tr>
<tr>
<td>25%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.122</td>
<td>0.238</td>
<td>0.349</td>
<td>0.455</td>
<td>0.556</td>
<td>0.652</td>
<td>0.745</td>
<td>0.833</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.022</td>
<td>0.038</td>
<td>0.049</td>
<td>0.055</td>
<td>0.056</td>
<td>0.052</td>
<td>0.045</td>
<td>0.033</td>
</tr>
<tr>
<td>50%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.143</td>
<td>0.273</td>
<td>0.391</td>
<td>0.500</td>
<td>0.600</td>
<td>0.692</td>
<td>0.778</td>
<td>0.857</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.043</td>
<td>0.073</td>
<td>0.091</td>
<td>0.100</td>
<td>0.100</td>
<td>0.092</td>
<td>0.078</td>
<td>0.057</td>
</tr>
<tr>
<td>75%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.163</td>
<td>0.304</td>
<td>0.429</td>
<td>0.538</td>
<td>0.636</td>
<td>0.724</td>
<td>0.803</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.063</td>
<td>0.104</td>
<td>0.129</td>
<td>0.138</td>
<td>0.136</td>
<td>0.124</td>
<td>0.103</td>
<td>0.075</td>
</tr>
<tr>
<td>100%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.182</td>
<td>0.333</td>
<td>0.462</td>
<td>0.571</td>
<td>0.667</td>
<td>0.750</td>
<td>0.824</td>
<td>0.889</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.082</td>
<td>0.133</td>
<td>0.162</td>
<td>0.171</td>
<td>0.167</td>
<td>0.150</td>
<td>0.124</td>
<td>0.089</td>
</tr>
<tr>
<td>150%</td>
<td>$CUE_{\text{actual}}$</td>
<td>0.217</td>
<td>0.385</td>
<td>0.517</td>
<td>0.625</td>
<td>0.714</td>
<td>0.789</td>
<td>0.854</td>
<td>0.909</td>
</tr>
<tr>
<td></td>
<td>$CUE_{\text{actual}}$ minus $CUE_{\text{apparent}}$</td>
<td>0.117</td>
<td>0.185</td>
<td>0.217</td>
<td>0.225</td>
<td>0.214</td>
<td>0.189</td>
<td>0.154</td>
<td>0.109</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY REFERENCES


