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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. Recognised 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. By incorporating these case-specific values in model simulations, we observed that the model projects up to 34% larger SOC stocks over a period of 64 years when non-growth metabolites are considered for estimating CUE, highlighting the crucial importance of accurate CUE quantification. Our considerations outlined here challenge the current ways how CUE is measured and we suggest improvements concerning the quantification of non-growth metabolites. 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 stabilisation 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 stabilised 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, providing a potential for C stabilisation. Recognised as a crucial control in C cycling, 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 (Allison et al., 2010; Bölscher et al., 2020; Frey et al.,

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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 non-growth anabolic C comprises all extracellular metabolites released from microbial cells into the surrounding soil, such as extracellular polymeric substances (EPS), exoenzymes, or nutrient mobilising compounds (Flemming and Wingender, 2010; Van Bodegom, 2007), and certain intracellular metabolites, such as storage compounds or endoenzymes (Mason-Jones et al., 2023, 2022, Fig. 1). Definitions of CUE and methods to quantify it determine which specific metabolites are ignored in the assessment of microbial 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 $^{13}$C or $^{14}$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 (Blazewicz and Schwartz, 2011; Canarini et al., 2019; 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. However, C directed towards the synthesis of non-growth metabolites 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 C stabilisation. 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) scrutinise 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 could 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 threefold: (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 negligible 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 conceptualisations 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., 2012, 2018). Depending on the organism and specific situation, these three

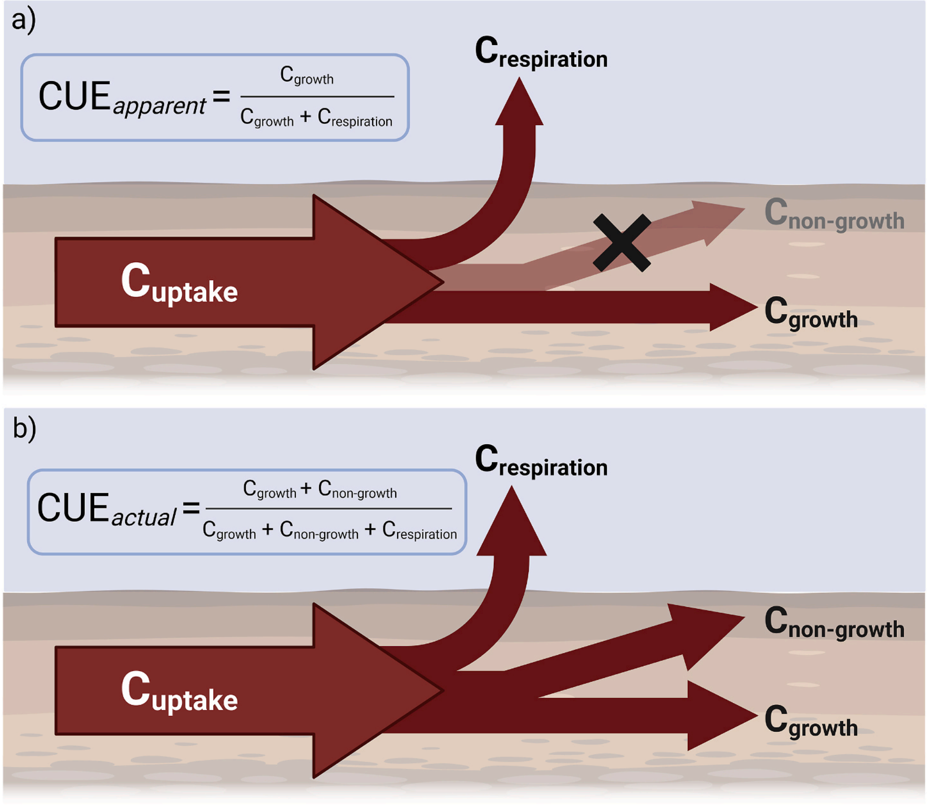

**Fig. 1.** Schematic illustration of carbon (C) partitioning during microbial metabolism when considering carbon-use efficiency (CUE). (**a**) Current concepts of CUE disregard C used for non-growth anabolism ($C_{non\text{-}growth}$). CUE is quantified from C used for growth ($C_{growth}$) and respiration ($C_{respiration}$) where the entire C uptake ($C_{uptake}$) is considered as the sum of $C_{growth}$ and $C_{respiration}$ (equation (1)). Current approaches measure therefore an apparent CUE ($CUE_{apparent}$). (**b**) To measure actual CUE ($CUE_{actual}$), $C_{non\text{-}growth}$ 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 stabilisation is of interest (equation (2)).

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 (also called growth yield; Frey et al., 2001; Herron et al., 2009; Sinsabaugh et al., 2013; Spohn et al., 2016; Thiet et al., 2006), as commonly used in microbiology (e.g. Gommers et al., 1988; Linton, 1991; von Stockar and Marison, 1993; see Supplementary Note for discussion on how neglecting non-growth anabolism affects estimations of growth efficiency). 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) may be 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 stabilisation 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. These additions are much higher compared to 0.012–216 μg glucose-C $g^{-1}$ soil, quantities common for unamended soils; 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 quantification is thus solely founded on experiments favouring growth over non-growth anabolism. These experiments investigated CUE under conditions with unrealistically high availability of low-molecular-weight substrate while microbial access to C is commonly limited in soil. 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 promoted the development of methods independent of $^{13}$C- or $^{14}$C-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 neglection 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., 2019, 2023; Redmile-Gordon et al., 2014, 2015). 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 non-growth anabolism not only affects the concept of CUE (i.e. it considers 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 independent from the adopted approaches (i.e. substrate-specific or substrate-independent CUE). In both approaches, the total C metabolised 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):

$$CUE_{apparent} = \frac{C_{growth}}{C_{growth} + C_{respiration}} \tag{1}$$

Where $CUE_{\text{apparent}}$ is the apparent CUE and $C_{growth}$ and $C_{respiration}$ 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 metabolised 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 stabilisation, we propose that *actual* CUE should be calculated, considering growth and non-growth anabolism (Fig. 1b):

$$CUE_{actual} = \frac{C_{growth} + C_{non-growth}}{C_{growth} + C_{non-growth} + C_{respiration}} \tag{2}$$

Where $CUE_{actual}$ is the actual CUE and $C_{non\text{-}growth}$ 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 two examples (i.e. extracellular enzymes, Domeignoz-Horta et al., 2023; EPS, Olagoke et al., 2022).

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

We employed two case studies to quantify potential underestimation of $\text{CUE}_{\text{actual}}$ 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 (section 4.3). In all cases, $\text{CUE}_{\text{actual}}$ versus $\text{CUE}_{\text{apparent}}$ is 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 $\text{CUE}_{\text{actual}}$ and $\text{CUE}_{\text{apparent}}$ when accounting for microbial C-investments into exoenzyme synthesis (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. Here, we estimated the C costs by considering the exoenzymes' capacities to process substrate molecules (BRENDA, 2023; Chang et al., 2021), along with previously measured enzyme turnover (Schimel et al., 2017), and the fraction of C present in the amino-acids constituting the enzymes (NCBI, 2023; Supplementary Methods, Table S1). In a second scenario (*enzyme pool expansion*), we assumed that the microbial community expands the initial exoenzyme pool by 20% within 24 h (Table S1). Similar increases in enzyme pools were observed previously following shifts in soil nutrient inputs (Allison and Vitousek, 2005).

Depending on the underlying scenario (Fig. 2, Table S2), our analysis demonstrates that exoenzyme production can influence CUE to contrasting degrees. For the first scenario, enzyme pool maintenance, the underestimation of $\text{CUE}_{\text{actual}}$ is minute, i.e. less than 0.01 differences between $\text{CUE}_{\text{actual}}$ and $\text{CUE}_{\text{apparent}}$ (Fig. 2a and b). This 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 $\text{CUE}_{\text{actual}}$. The assumed 20% increase in the exoenzyme pool caused underestimations of $\text{CUE}_{\text{actual}}$ between 0.002 and 0.189

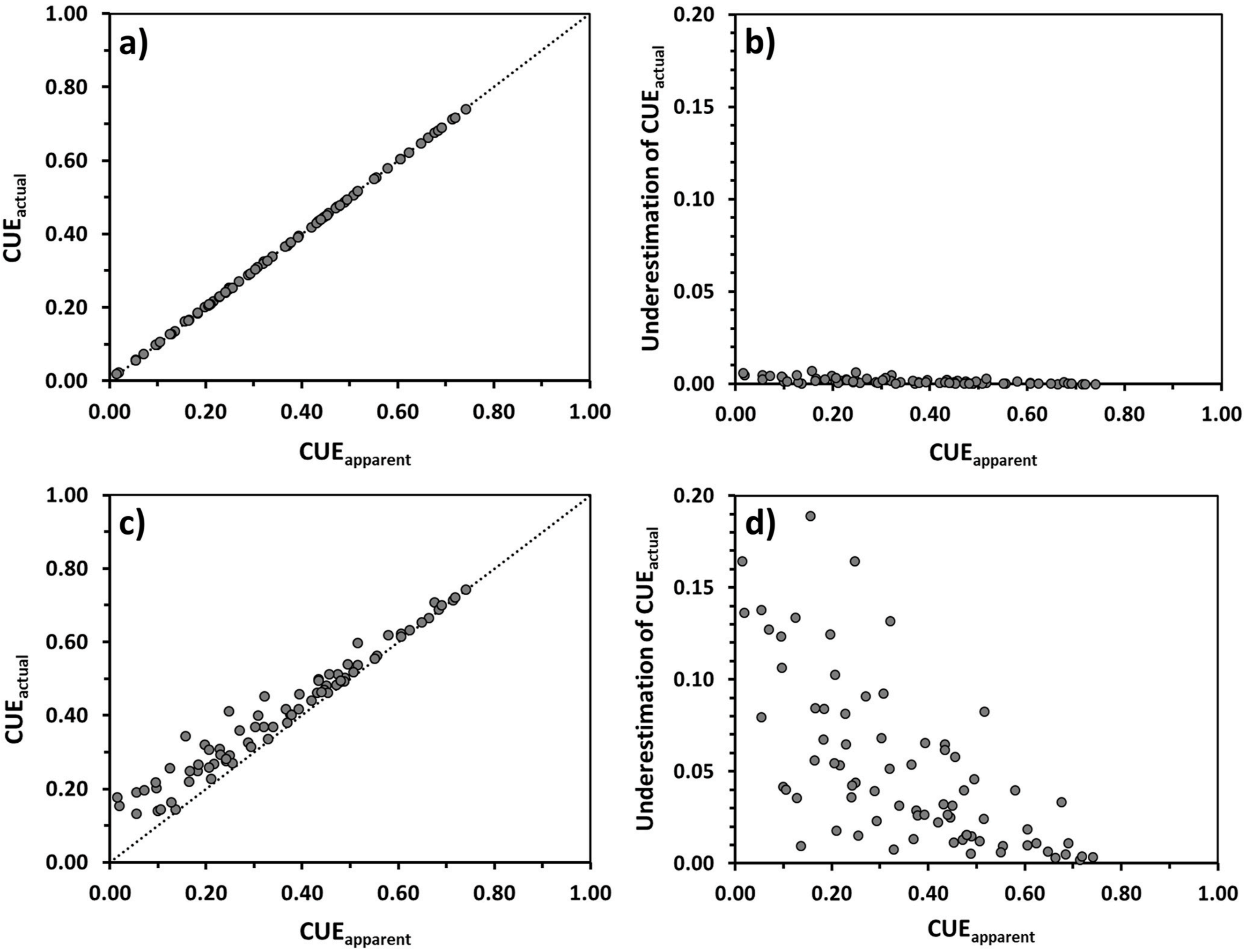

**Fig. 2.** Actual and apparent carbon-use efficiencies (CUE$_{\text{actual}}$ and CUE$_{\text{apparent}}$, respectively) considering extracellular enzyme formation based on data adopted from Domeignoz-Horta et al. (2023). The displayed results consider two scenarios for enzyme formation: First, microbial 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$_{\text{apparent}}$ (equation (1)) and corresponding CUE$_{\text{actual}}$ (equation (2)) for the two scenarios, respectively. The dotted lines indicate the 1:1 ratio of equal CUE$_{\text{apparent}}$ and CUE$_{\text{actual}}$. Right panels (**b**, **d**) present the underestimation of CUE$_{\text{actual}}$ (i.e. CUE$_{\text{actual}}$ minus CUE$_{\text{apparent}}$) plotted as function of assumed CUE$_{\text{apparent}}$ for the two scenarios, respectively.

(Fig. 2c and d). Here, 40% of the samples resulted in an underestimation of 0.05 or larger and CUE$_{\text{actual}}$ was more than twice as high as CUE$_{\text{apparent}}$ in 12% of the samples. Consequently, even smaller increases in the exoenzyme pool than the assumed 20% could result in a considerable underestimation of CUE$_{\text{actual}}$. These findings demonstrate that microorganisms can potentially invest an important fraction of their C budget into the production of enzymes, thereby influencing CUE. We therefore consider that microbial C investments into enzymes should not be—a priori—ignored during investigations of CUE.

The high sensitivity of 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 advance our understanding of the physiological responses of microorganisms to changes in environmental conditions which may either lead to maintenance or increases of 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 how far these coupled—yet shifted in time—changes affect the underestimation of CUE$_{\text{actual}}$ over time. Yet, due to the time-shift, it can be assumed that CUE$_{\text{actual}}$ remains high over longer periods than CUE$_{\text{apparent}}$, because CUE$_{\text{apparent}}$ is only affected by accelerated growth while CUE$_{\text{actual}}$ increases already with the preceding increase in enzyme formation.

In the second scenario, exoenzyme pool expansion, the underestimation of CUE$_{\text{actual}}$ was dependent on measured CUE$_{\text{apparent}}$. We found a clear trend that the underestimation of CUE$_{\text{actual}}$ decreased with increasing CUE$_{\text{apparent}}$ (Fig. 2d). This trend occurs, because the relative distribution of C between metabolic pathways affects CUE. If the same amount of C is used for enzyme production across different CUE$_{\text{apparent}}$, adding C$_{\text{non-growth}}$ to the CUE equation will have a stronger effect on CUE$_{\text{actual}}$ when CUE$_{\text{apparent}}$ (and thus growth) is low. This is due to the fact that the relative contribution of C$_{\text{non-growth}}$ to the total anabolic C use is high compared to cases when CUE$_{\text{apparent}}$ is high (see also section 4.3). Commonly, substrate-independent methods (i.e. $^{18}$O tracing techniques) measure CUE$_{\text{apparent}}$ for SOC decomposition less than 0.40 (Geyer et al., 2019). As exoenzyme-related underestimations of CUE$_{\text{actual}}$ seem to be larger below this value, special considerations should be given to potential effects of exoenzyme pool dynamics on CUE when substrate-independent methods are applied.

### 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 and 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 Tables S3 and S4). 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, $CUE_{actual}$ was 0.52 or 0.62 compared to $CUE_{apparent}$ of 0.40 or 0.50, respectively (Fig. 3a). The implication of these underestimations becomes clearer when we consider the relative differences between $CUE_{actual}$ and $CUE_{apparent}$. In our case, $CUE_{actual}$ would be 4–30% higher than $CUE_{apparent}$, which means that 4–30% more C may remain in soil than estimated in the approach neglecting microbial non-growth anabolism.

Furthermore, in all treatments, underestimations of $CUE_{actual}$ peak in a range of $CUE_{apparent}$ between 0.40 and 0.50 and decrease towards both ends of the CUE range (i.e. 0.10 to 0.80, Fig. 3b). Yet, underestimations of $CUE_{actual}$ can remain high even at both ends of the range of $CUE_{apparent}$ (i.e. as high as 0.05 and 0.07 for $CUE_{apparent}$ of 0.10 and 0.80, respectively; Fig. 3b). As consequence, $CUE_{actual}$ could be substantially underestimated over the entire range of usually reported CUEs in soils (i.e. <0.40 for substrate-independent CUE and <0.60 for substrate-specific CUE; Dijkstra et al., 2015; Geyer et al., 2019; Sinsabaugh et al., 2013) and the range of CUE usually assumed in soil C models (i.e. 0.15–0.60; Manzoni et al., 2012). In the range of $CUE_{apparent}$ commonly measured for native SOC (i.e. CUE <0.40; Geyer et al., 2019), underestimations of $CUE_{actual}$ were as high as 0.12 (Fig. 3b).

Underestimations of $CUE_{actual}$ seem to depend on the metabolised substrate. While we found relatively small underestimations of $CUE_{actual}$ 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; Fig. 3b). The differences were related to the relative production of EPS to biomass (Fig. 3c; Olagoke et al., 2022). Starch is a readily available substrate for microbial metabolisation, while cellulose breakdown is more complex, and control soils were depleted of labile and particulate SOC (Olagoke et al., 2022). If the here revealed substrate-dependent underestimations of $CUE_{actual}$ are generally true, underestimations of $CUE_{actual}$ 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 ceasing hot-spots of microbial activity with increased cell lysis (Kuzyakov and Blagodatskaya, 2015). Yet, underestimations of $CUE_{actual}$ 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 $CUE_{actual}$ 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).

### 4.3. *Theoretical approach: what are the general effects of neglecting non-growth anabolism for CUE?*

Our aim here was to evaluate the quantity of non-growth metabolites required to cause substantial underestimation of $CUE_{actual}$. Because quantitative information on non-growth metabolites in soils is limited, we deployed a theoretical approach using various relative microbial C allocation ratios between non-growth and growth anabolism as well as ratios between non-growth anabolism and C uptake (see Supplementary Methods). In general, underestimations of $CUE_{actual}$ increase with increasing amounts of C used for non-growth anabolism (Fig. 4 Table S5). They can be above 0.05 when the C used for non-growth anabolism is ≥ 5% of C uptake (Fig. 4a) or ≥25% of the C used for growth. Underestimations of $CUE_{actual}$ increase with decreasing $CUE_{apparent}$ for constant ratios of non-growth anabolism to C uptake (Fig. 4a). For ratios of non-growth to growth anabolism, graphs follow inverse U-shapes peaking around a $CUE_{apparent}$ of 0.35–0.50 (Fig. 4b). The inverse U-shapes are caused by the balance (or imbalance) of the C allocation between the variables $C_{respiration}$ and $C_{growth}$ (equation (1)): At low

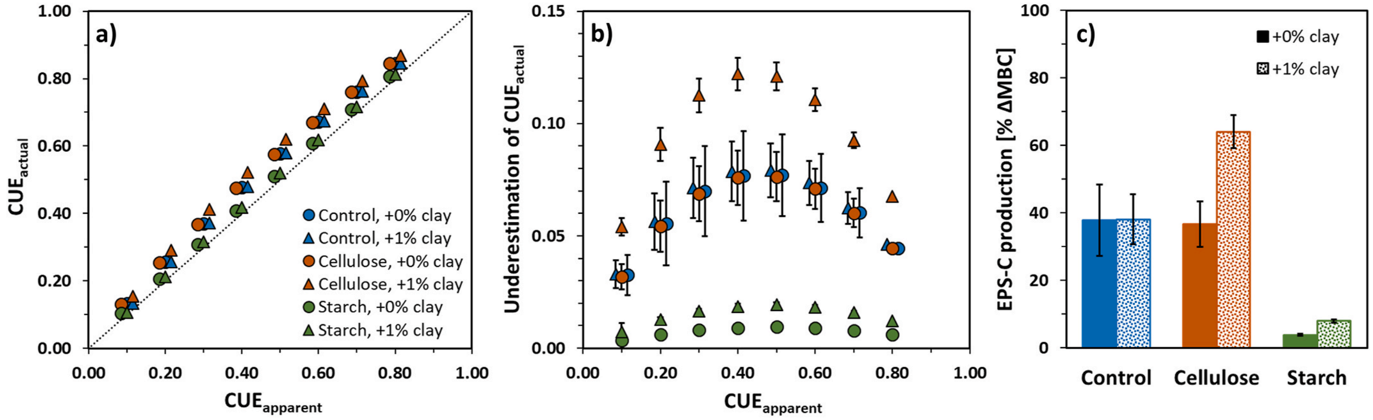

**Fig. 3.** Actual and apparent carbon-use efficiencies ($CUE_{actual}$ and $CUE_{apparent}$, respectively) and production of extracellular polymeric substances (EPS) calculated from data of Olagoke et al. (2022). (**a**) Comparison between assumed $CUE_{apparent}$ (equation (1)) and corresponding $CUE_{actual}$ (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 $CUE_{apparent}$ and $CUE_{actual}$ (**b**) Underestimation of $CUE_{actual}$ (i.e. $CUE_{actual}$ minus $CUE_{apparent}$) plotted as a function of assumed $CUE_{apparent}$. (**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.

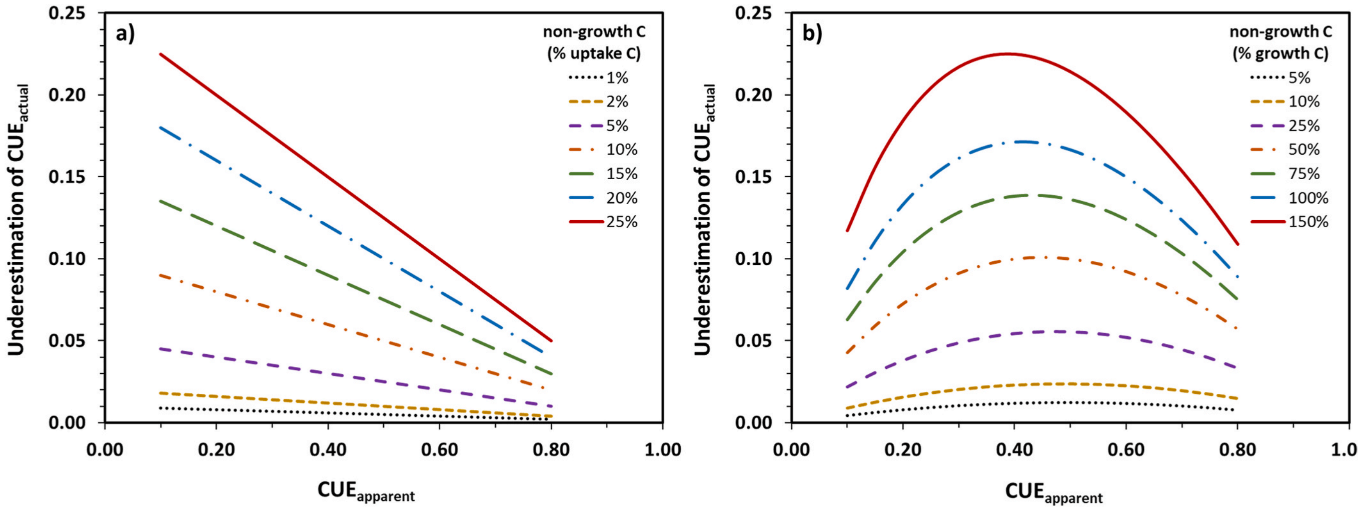

**Fig. 4.** Underestimation of actual carbon-use efficiency (CUE$_{actual}$) in relation to (**a**) microbial carbon (C) uptake and (**b**) microbial C allocation between non-growth and growth anabolism. Underestimation of CUE$_{actual}$ (i.e. CUE$_{actual}$ minus CUE$_{apparent}$) is plotted as function of assumed apparent CUE (CUE$_{apparent}$). CUE$_{apparent}$ was assumed to range between 0.10 and 0.80. CUE$_{actual}$ was calculated for fourteen scenarios with C used for non-growth anabolism relative to (**a**) C uptake ranging from 1 to 25% of C uptake and (b) C used for growth ranging from 5 to 150% of C used for growth.

CUE$_{apparent}$, most of the total C used is directed towards respiration and C$_{respiration}$ dominates the CUE calculations. Adding C$_{non\text{-}growth}$ to the calculation has therefore a relatively small effect on CUE. At high CUE$_{apparent}$, most of the C is used for growth. Similarly, as C$_{growth}$ dominates the CUE calculation, adding C$_{non\text{-}growth}$ to the calculation has a relatively small effect. Considering C$_{non\text{-}growth}$ for CUE has the largest effect when the distribution of C between respiration and growth is balanced. Our theoretical approach therefore demonstrates that non-growth anabolism should be integrated in CUE quantifications in situations when non-growth C is equal or more than 5% of C uptake or 25% growth C.

## 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 a step-wise increase in the model's organic matter retention coefficient (ε, which can be seen as a proxy for CUE), covering the underestimations of CUE$_{actual}$ revealed in the case studies and theoretical approach (see Supplementary Methods). Our modelling approach provides a sensitivity analysis. Projected SOC stocks need to be interpreted in this context.

In the model reference scenario that assumed no underestimation of CUE$_{actual}$ (i.e. CUE = 0.14), SOC stocks are modelled with 4.21 kg C m$^{-2}$. Yet, for the largest difference in CUE (i.e. underestimation of CUE$_{actual}$ being 0.23, non-growth anabolic C equal to 25% of C uptake or 150% of growth C, see red solid lines 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 study, differences in CUE$_{actual}$ and CUE$_{apparent}$ ranged from no differences up 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 0.004–0.12 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).

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

## 6. Perspective on microbial CUE in the light of SOC stabilisation

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

Here, we demonstrate that non-growth anabolites can make up a substantial part of microbially processed C, thus affecting microbial CUE (Figs. 2–4). The findings of our case studies (section 4) are supported by other (semi-)quantitative investigations. First, non-growth conditions are expected to dominate in soil, where access to available substrate and nutrients is restricted (Hobbie and Hobbie, 2013; Joergensen and Wichern, 2018; Kuzyakov and Blagodatskaya, 2015). While the non-growth state of microorganisms remains largely uncharacterised with 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 that 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 h incubation experiment (Schmalenberger et al., 2015). These examples represent a glimpse of studies illustrating that soil microbial communities produce a diverse set of non-growth metabolites, potentially in quantities that can be crucial when estimating CUE. Non-growth metabolites should thus

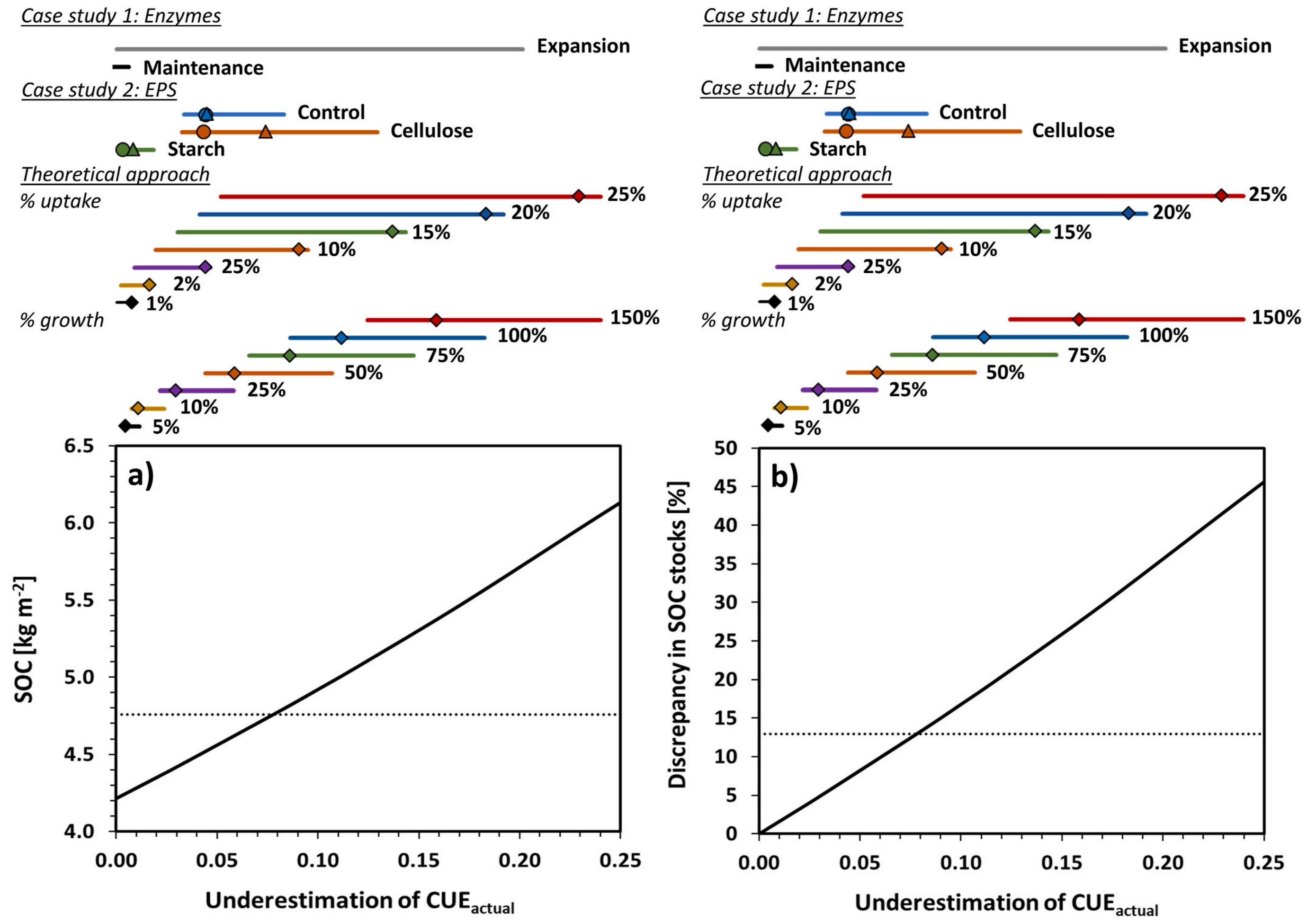

**Fig. 5.** Results from the modelling approach showing soil organic carbon (SOC) stocks calculated for the Green Manure treatment of the Ultuna Long-Term Soil Organic Matter Experiment (Herrmann and Witter, 2008; Persson and Kirchmann, 1994) in the year 2020. The SOC stocks were modelled assuming carbon-use efficiencies (CUE) 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 CUE$_{actual}$) are shown on the right. Horizontal solid lines above the graphs show the range of the underestimation of CUE$_{actual}$ 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 CUE$_{apparent}$ 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.

not be neglected, 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 soil may be as stable as microbial growth residues and foster processes that promote C stabilisation, 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., 2007, 2015, 2021; 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 stabilisation via soil aggregation (Guhra et al., 2022). These examples demonstrate that non-growth anabolism does not only serve important functions for microbial survival but likely contributes to stabile SOC in similar ways as residues of microbial growth. Recent findings indicate even that products of non-growth anabolism may be more important in 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 stabilisation, 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 the dynamics of most non-growth metabolites (such as EPS, enzymes and osmolytes) are coupled and alter interrelatedly 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 scarce (Olagoke et al., 2022). Such consecutive and interrelated dynamics of metabolite production over time and space have implications for CUE measurements. In our example, $CUE_{apparent}$ would only increase for a short time with enhanced microbial growth, while $CUE_{actual}$ 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 the identification of metabolic C fluxes that quantify endo- and exogenous metabolites in soil. Over the last years, advances have been made to quantify various microbial metabolites in soil. Different extraction techniques for microbial EPS have been tested and a method was described which allows measuring changes in extracellular polysaccharides and proteins over a short time (Olagoke et al., 2022; Redmile-Gordon et al., 2014). Although we still lack procedures to quantify all types of storage compounds, protocols are readily available for key lipid forms of microbial energy storage (Mason-Jones et al., 2022) including triacylglycerides (Banfield et al., 2017; Gorka et al., 2023; Mason-Jones et al., 2023) and polyhydroxyalkanoates (Mason-Jones et al., 2019). In conjunction with $^{13}$C- or $^{2}$H-labelling, these protocols allow the quantification of small changes in microbial storage over a short time and in combination with substrate-specific and substrate-independent CUE, respectively (Canarini et al., 2023; Mason-Jones et al., 2023). Saccharide storage compounds (i.e. glycogen and trehalose; Mason-Jones et al., 2022) could be targeted using a protocol for glycogen extraction from soil (Bölscher et al., 2016) in combination with the chloroform fumigation extraction method (Vance et al., 1987). Yet, its feasibility and sensitivity for quantifying changes in saccharide storage compounds remain to be examined. While we describe a possibility to estimate extracellular enzyme C (see Supplementary Methods), metabolomics offers a way forward to quantify low molecular weight metabolites (Johns et al., 2017; Patti et al., 2012; Swenson et al., 2015; Withers et al., 2020). Metabolomics can quantify exo- and/or endometabolites (Swenson et al., 2015). Especially untargeted metabolomics with the capability to measure a wide range of low molecular weight metabolites (Patti et al., 2012; Swenson et al., 2015; Withers et al., 2020) bears potential to gain more accurate estimations of $CUE_{actual}$. Beyond providing more accurate quantifications of $CUE_{actual}$, the combination of CUE measurements with metabolomics could provide additional insights into underlying microbial functioning, especially when combined with other "omics" technologies (e.g. genomics and transcriptomics; Chowdhury et al., 2021; Daniel, 2005; Withers et al., 2020). In addition to group-specific metabolite quantification, $^{13}$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., 2011, 2015, 2022). $^{13}$C Metabolic Flux Analysis may therefore offer great potential for opening the black boxes of soil microbial physiology and CUE, especially when combined with other techniques described above.

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). However, we will need to understand the underlying processes of microbial physiology to judge in which situations 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 (Le 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 $CUE_{actual}$, 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 stabilisation, non-growth anabolism is essential and needs to be quantified to capture the 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 the CUE range commonly observed in soils. Considering an exoenzyme pool expansion by 20% resulted in a 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. These underestimations of CUE and SOC stock are case and model-specific. Future work is needed to test whether similar underestimations occur under different environmental conditions and *in-situ*. Yet, our results emphasised that substantial underestimations of CUE are feasible when non-growth metabolites are ignored. 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. As revealed by multiple studies, microbial communities can invest a substantial amount of metabolised C into non-growth metabolites, which are likely as much stabilised in soils as residues of microbial growth. Both, non-growth and growth residues, are exposed to the same C stabilisation 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 stabilised 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 the terrestrial C cycle.

## CRediT authorship contribution statement

**Tobias Bölscher:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Cordula Vogel:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation,

Visualization, Writing – original draft, Writing – review & editing. **Folasade K. Olagoke:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Katharina H.E. Meurer:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Anke M. Herrmann:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Tino Colombi:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Melanie Brunn:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Luiz A. Domeignoz-Horta:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anke M. Herrmann has been a member of the editorial board of Soil Biology & Biochemistry until spring 2022.

## Data availability

We used priviously published data. These data will be made available upon request to the authors of the original study.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2024.109400.

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