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"A novel approach to measure microbial carbon use efficiency by estimating growth from ^{18}O incorporation into DNA"

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I. GENERAL INTRODUCTION

1. Microbial physiology and CUE

The potential of soils to store carbon is a major mechanism that could contribute to mitigate the increase of atmospheric CO_2, and consequently global warming, in the wake of climate change (Cox *et al.*, 2000; Davidson & Janssens, 2006). Soil carbon storage can occur via physical or chemical mechanisms that protect plant litter from degradation; usually, however, it is mediated by decomposition by the microbial soil community (Cebrian & Lartigue, 2004). Bacteria, fungi and archaea depolymerize complex plant-derived compounds via excretion of extracellular enzymes and take up the mono- or oligomeric products to meet their energy and nutritional demands. Four possible fates await carbon compounds after uptake by a microorganism: they can either (a) be respired to CO_2, (b) be used for the production of new biomass (growth), (c) be excreted, for example in the form of extracellular enzymes or intermediate metabolites, or (d) be stored in the cell (van Bodegom, 2007; Manzoni *et al.*, 2012). Of these possibilities, only the carbon that is invested into new biomass generally has the possibility to be stabilized in soil for longer time scales, for example in the form of microbial cell wall components (Six *et al.*, 2006; Schimel & Schaeffer, 2012). The efficiency with which microorganisms utilize carbon compounds for the production of new biomass, which is called carbon use efficiency (CUE), growth efficiency or growth yield, is therefore an important parameter for the prediction of soil carbon storage in a future climate (Bradford & Crowther, 2013).

Microbial carbon use efficiency is defined as the ratio of carbon that is used for the production of new biomass relative to the total carbon that is taken up, and thus describes how anabolic and catabolic reactions are balanced (del Giorgio & Cole, 1998; Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). For methodological reasons, measurement of carbon uptake is often simplified as the sum of carbon used for growth and respired carbon (Sinsabaugh *et al.*, 2013). CUE is variable: it can be different for different groups of microorganisms and also varies with environmental conditions, substrate quality and nutrient status, providing microorganisms with the ability to adjust their carbon utilization to the environment (del Giorgio & Cole, 1998). A high CUE implies that a large fraction of the consumed carbon is converted into new microbial biomass, whereas a low CUE implies a high loss of carbon via respiration as CO_2 (Manzoni *et al.*, 2012).

Carbon excretion and storage are usually assumed to be negligible compared to growth and respiration. Carbon compounds that are excreted from the cell include diverse compounds with very different functions, such as extracellular enzymes, metabolites or polysaccharides (del Giorgio & Cole, 1998). While generally low compared to respiration and growth, exudation of carbon compounds can be high under certain circumstances, such as

anaerobic conditions or a high substrate carbon excess in relation to the microorganism's nutrient requirements (Manzoni *et al.*, 2012). Excreted carbon compounds are usually not included for the estimation of growth in the context of CUE. Firstly, the most common methods do not measure excreted carbon as a part of newly produced biomass, as growth is estimated either by determining the amount of carbon incorporated into cells or by measuring DNA or protein production (del Giorgio & Cole, 1998; Manzoni *et al.*, 2012). Secondly, there is also a conceptual reason for its exclusion from the growth term: generally speaking, carbon compounds that are excreted do not, if only because of their chemical composition, have the potential for long-term stabilization in soils (Manzoni *et al.*, 2012). Carbon storage in the cell, on the other hand, is included in growth when it is measured with the most common method used in soils (a biomass-based method based on incubation with a ^{13}C-labeled substrate), whereas it is excluded by the conventional method for aquatic systems (see below). As similar considerations apply as for carbon excretion, storage may best be excluded from growth.

The theoretical range of CUE, as a ratio, is between 0 (all consumed carbon is respired) and 1 (all consumed carbon is used for growth). Negative values are also possible if the carbon compounds that are consumed do not provide sufficient energy or carbon to satisfy the microorganism's maintenance requirements, such as turnover of macromolecules, defense mechanisms, and osmoregulation (van Bodegom, 2007; Sinsabaugh *et al.*, 2013). In practice, a CUE value of 1 is unobtainable, since part of the consumed carbon must be used to gain energy for growth and maintenance. Based on thermodynamic considerations about the maximum gain of ATP and electrons, the highest achievable CUE value has been calculated at 0.6 (Sinsabaugh *et al.*, 2013). Since environmental conditions will not usually be ideal for growth, realized CUE values of natural microbial communities are expected to be considerably lower, with an average between 0.2 and 0.3 (Sinsabaugh *et al.*, 2013). Nevertheless, the average value reported for terrestrial systems is 0.55, and thus close to the thermodynamic maximum (Sinsabaugh *et al.*, 2013). Comparing ten studies of soils with strong environmental or nutrient gradients, Manzoni *et al.* (2012) reported CUE values ranging from 0.10 to 0.83. Indeed, quite a larger number of studies find values for CUE in soil that are well above the thermodynamic maximum (e.g. Nguyen & Guckert, 2001; Šantrůčková *et al.*, 2004; Ziegler *et al.*, 2005; Thiet *et al.*, 2006). By contrast, measured CUE values in aquatic systems are generally much lower and average between 0.2 and 0.3 (del Giorgio & Cole, 1998; Manzoni *et al.*, 2012). This striking divergence between terrestrial and aquatic systems is probably not due to physiological or environmental differences, but caused by different methodologies for the measurement of CUE (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013), as will be explained in more detail below.

2. Controls on CUE

The body of literature that has developed on the subject of CUE in natural environments indicates that the CUE of microbial soil communities is controlled by the quality and elemental composition of substrates, community composition, and environmental factors such as temperature (del Giorgio & Cole, 1998; Manzoni *et al.*, 2012). Yet, although the effects of these parameters have been studied for quite some time, no consensus has been reached so far concerning their importance for microbial CUE. This is partly due to different studies using different methods to determine CUE, which makes it difficult to compare the results, in particular between aquatic and terrestrial systems. Another reason lies in the fact that it is not completely possible to distinguish between the various controlling factors when conducting a study in a natural environment; for example, a temperature gradient can go hand in hand with a nutrient gradient, one factor thus masking the effect of the other (Manzoni *et al.*, 2012).

Temperature. Temperature has been identified early on as a potential control on CUE. Both growth and respiration rates generally increase with temperature; they are however not affected to the same extent. While microbial growth is generally positively correlated with temperature, it follows an optimum relationship (Apple *et al.*, 2006). By contrast, no optimum has been found for respiration: under environmental conditions, respiration increases with temperature without levelling off at a certain threshold, and it is more strongly affected by temperature than growth. The net effect is a reduction in CUE with increasing temperature (Apple *et al.*, 2006; Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). Some studies, however, did not find a temperature effect on CUE (Dijkstra *et al.*, 2011; Hagerty *et al.*, 2014). This might again be partly explained by the methods that are used to determine CUE in soils. Frey *et al.* (2013) showed that CUE was only marginally affected by temperature in soils amended with simple substrates such as glucose, whereas it declined strongly at increasing temperatures when more complex substrates were used. The standard method for the determination of CUE in soils is based on the incubation with a labile substrate, which could result in an attenuated temperature effect. Hagerty *et al.* (2014), on the other hand, argued in a recent study that the temperature sensitivity of CUE observed in many studies could arise from confounding decreased CUE with accelerated microbial turnover when the time of measurement of CUE is too long. In a laboratory study of two soils that were incubated at different temperatures, they observed no change in CUE with increasing temperature in the short term (hours), but an increase in respiration at constant microbial biomass. They concluded that the apparent decrease of CUE with temperature that has been found in other studies might in fact be caused by an acceleration of microbial turnover. Finally, it should be

noted that temperature might co-vary with nutrient availability or substrate quality, and the temperature effects observed in some studies might thus not be attributable to temperature alone (Manzoni *et al.*, 2012; Frey *et al.*, 2013; Sinsabaugh *et al.*, 2013).

Soil moisture. Microbial growth declines at low soil water, as microorganisms become increasingly isolated from their substrates and a higher proportion of the community may enter a state of dormancy (Iovieno & Baath, 2008). In addition, drought conditions can cause microbes to invest into the synthesis of osmoregulatory compounds and mucopolysaccharides at the expense of growth (Schimel *et al.*, 2007; Tiemann & Billings, 2011). Increased microbial maintenance demands and attenuated growth may thus result in decreased CUE under water stress conditions (Manzoni *et al.*, 2012). However, when CUE is determined by incubation with a ^{13}C-labeled substrate, the storage of osmolytes under drought conditions might cause a short-term increase in (apparent) CUE (Herron *et al.*, 2009; Sinsabaugh *et al.*, 2013). On the other end of the scale, water saturation of soils has also been shown to decrease CUE. Under anaerobic conditions prevalent in water-saturated soils, microorganisms cannot oxidize substrates completely and consequently gain less energy per unit substrate that is consumed, while the partly oxidized metabolites are excreted from the cell (Šantrůčková *et al.*, 2004; Manzoni *et al.*, 2012). Lastly, repeated changes in water availability (drying-rewetting cycles) can also decrease CUE, because microbes have to re-adapt to changing water conditions by frequent synthesis and release of osmolytes, resulting in increased energy and carbon requirements for maintenance (Tiemann & Billings, 2011).

Substrate stoichiometry, nitrogen and carbon limitation. According to stoichiometric theory, decomposing microorganisms have to maintain their elemental composition within a narrow range, independently of the elemental composition of the substrates they consume (Manzoni & Porporato, 2009; Sinsabaugh *et al.*, 2013; Mooshammer *et al.*, 2014a). In terrestrial systems, the element that is usually limiting is nitrogen, and the most extensively studied aspect of substrate stoichiometry is therefore the ratio of carbon to nitrogen (C:N). The threshold element ratio (TER), or critical element ratio, is the ratio of optimum elemental substrate composition for growth and defines the C:N ratio at which carbon and nitrogen are co-limiting. Nitrogen is limiting when substrate C:N exceeds the TER, carbon is limiting when it falls below TER (Manzoni *et al.*, 2012). The TER is positively correlated with microbial biomass C:N and negatively correlated with CUE, but also appears to scale with the initial litter C:N ratio (Manzoni *et al.*, 2008). In terrestrial systems, it is usually quite constant at a value between 20 and 25. Litter C:N, on the other hand, is rather variable, with a global average between 50 to 70 (Sinsabaugh *et al.*, 2013), whereas soil organic matter has a C:N

ratio between 14 and 17 (Xu *et al.*, 2013; Zechmeister-Boltenstern *et al.*, 2015). The typical situation in soils is therefore one of low C:N relative to the TER, i.e. carbon limitation of the decomposer community. By contrast, litter C:N ratios are usually above the TER, and thus nitrogen is the limiting element in the initial stages of litter decomposition. Microorganisms can respond to such limitations by disposal of either the excess nitrogen (lowering their nitrogen use efficiency, with no effect on CUE) (Mooshammer *et al.*, 2014b) or the excess carbon in the substrate. The latter is done by excretion or so-called "overflow" respiration (Schimel & Weintraub, 2003) and will result in a decrease of CUE (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). Contrary to a high substrate C:N, low C:N leads to a tight coupling between catabolism and anabolism and subsequently a high CUE (del Giorgio & Cole, 1998). However, when carbon supply reaches very low levels, microbes will use the available carbon to meet their maintenance demands rather than for growth, ultimately resulting in a decline of CUE (Sinsabaugh *et al.*, 2013). It is worth noting that CUE cannot always be predicted from the stoichiometric imbalance between microbial community and substrates alone. For example, some microorganisms may preferentially decompose substrates with low C:N, thereby maintaining a higher CUE than predicted by the bulk litter C:N (Sinsabaugh *et al.*, 2013). Notably, Kaiser *et al.* (2014) suggested that stoichiometric imbalances between litter and microbial community might be alleviated by microbial community dynamics that result in different turnover rates for nitrogen and carbon (see below).

Substrate quality. Aside from elemental stoichiometry, other chemical properties of the substrate, such as chemical structure and degree of reduction, can also influence CUE. The degradation of complex compounds requires a larger number of enzymatic reactions than that of labile compounds. It will therefore result in greater carbon losses, e.g. because of higher respiratory costs for enzyme production, reducing the amount of carbon that can be used for growth and consequently lowering CUE (Manzoni *et al.*, 2012). The second important aspect of a substrate's quality is its degree of reduction, which is defined as the number of electron equivalents per mole carbon. Relatively oxidized compounds have a low degree of reduction and less energy can be gained from their decomposition. The degradation of highly reduced substrates therefore enables microorganisms to maintain a higher CUE than the degradation of substrates with a lower degree of reduction (del Giorgio & Cole, 1998). Substrate quality in soils also changes with time: as decomposition proceeds, labile and highly reduced compounds are exhausted and the remaining substrate pool becomes increasingly difficult to decompose. Increased energy efforts to degrade substrates will, for example, reduce CUE in aging litter communities (Ziegler *et al.*, 2005; Manzoni *et al.*, 2012).

Community composition and dynamics. In recent years, an increasing number of studies have drawn attention to the fact that not only environmental factors and substrate properties, but also properties of the microbial community itself may influence CUE. Soil microbial communities are composed of different functional groups of organisms exhibiting various life-history strategies, e.g. with regard to turnover and biomass C:N ratio (Kaiser *et al.*, 2014), which will influence their CUE. As the microbial community composition varies from soil to soil, the differing CUEs of different microbial groups will result in different community CUEs for each soil. For example, it has been shown that opportunistic, fast-growing microbial communities (r-strategists) exhibit high growth rates but low CUE values, whereas slow-growing communities that are adapted to low substrate concentrations (K-strategists) have low growth rates at high CUEs (Shen & Bartha, 1996; Manzoni *et al.*, 2012). On a very coarse phylogenetic level, the distinction between fungi and bacteria could also be important: fungi have a higher and more variable C:N ratio than bacteria, slower turnover rates, and are assumed to decompose complex substrates that are largely inaccessible to bacteria, such as lignin (Rousk & Bååth, 2011). However, early assumptions that high fungi to bacteria ratios in soils would directly result in increased CUEs were refuted in later studies (Six *et al.*, 2006; Thiet *et al.*, 2006; Manzoni *et al.*, 2012). Nevertheless, indirect effects of the fungi to bacteria ratio are probable: since fungi have a higher biomass C:N ratio than bacteria, they will be less sensitive to nitrogen limitation. For instance, a study by Keiblinger *et al.* (2010) showed that the CUE of fungi in culture increased with increasing substrate C:N, whereas bacterial CUE declined. Moreover, a recent modeling study (Kaiser *et al.*, 2014) demonstrated that community dynamics may be able to buffer the effects of resource stoichiometry on CUE: in model simulations, the succession of microbes belonging to different functional groups led to the emergence of a high C:N substrate pool, which was turned over slowly, as well as a low C:N pool (microbial necromass), which was turned over at a faster rate. As a consequence, the C:N ratio of dissolved organic matter was relatively low, independent of the litter C:N ratio. This also resulted in a community CUE that stayed on a constantly high level, irrespective of the initial substrate C:N ratio.

3. CUE and global change

Global change – including elevated atmospheric CO_2 levels, rising temperatures, changed precipitation patterns, and increased nitrogen inputs into ecosystems by means of nitrogen deposition and fertilization – is expected to have a strong influence on CUE. CUE, in turn, is one of the main drivers of carbon sequestration in soils – a potential mechanism to buffer

atmospheric CO_2 and slow down global warming (Schimel & Schaeffer, 2012) – and as such taken into account by state-of-the-art biogeochemical models predicting the effects of climate change on the carbon cycle (Sinsabaugh *et al.*, 2013). Many models assume CUE to be constant and set it at values ranging from 0.15 to 0.6; some also distinguish between different pools of carbon (e.g. labile and recalcitrant pools) with different CUEs (Manzoni *et al.*, 2012). More recent modeling approaches, however, take into account that CUE may vary with environmental changes such as temperature or litter input and quality (Allison *et al.*, 2010; Frey *et al.*, 2013; Wieder *et al.*, 2013).

Predictions of the variation of CUE in the face of changing environmental conditions are complicated by the fact that there are still large gaps of knowledge concerning the controls of CUE, as described above. Even with regard to a relatively straightforward factor such as temperature, it is not yet entirely clear to what extent it influences CUE. In accordance with the most wide-spread opinion, CUE is expected to decrease with rising temperatures and with an increasing occurrence of water-stress events in the wake of climatic change (Tiemann & Billings, 2011; Manzoni *et al.*, 2012), but little is known about whether microbial community CUE may adapt to changing environmental conditions in the longer term. By contrast, increased nitrogen deposition will likely result in elevated CUE of soil microbial communities whose growth was previously limited by nitrogen supply (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). Elevated atmospheric CO_2 concentrations, on the other hand, might increase the C:N ratio of plant litter, thereby resulting in nitrogen limitation and reducing CUE (Manzoni *et al.*, 2012). Although most of these considerations suggest that CUE will decrease as a consequence of global change, it is difficult to predict how the projected responses of CUE will work together, influence each other, or to what extent they might cancel each other out. Changes in CUE can also have indirect effects that may serve as a negative or positive feedback mechanism. In addition, CUE might adjust to changed environmental conditions, and thus stay relatively constant or decrease less than might be expected. Several mechanisms are possible to achieve such an adjustment. The microbial community composition might change as a result of competitive or evolutionary processes that favor microorganisms with a high CUE; alternatively, physiological changes could re-balance anabolic and catabolic reactions in a way that stabilizes CUE (Allison *et al.*, 2010; Wieder *et al.*, 2013).

The response of CUE to climatic change could induce either a positive (reinforcing) or negative (self-regulating) feedback, depending on the direction of a change in CUE and its adjustability. For global warming, Allison *et al.* (2010) developed a microbial-enzyme model to study the potential on the soil organic carbon (SOC) pool mediated by CUE. Under increasing temperatures, a constant CUE resulted in drastic carbon losses of the SOC pool

to the atmosphere. This effect was caused by increased biomass and enzyme activity that provided a positive feedback by increasing decomposition and CO_2 efflux to the atmosphere. If, on the other hand, CUE declined with temperature, microbial biomass would decline as well, resulting in only moderate decreases of the SOC pool or even long-term increases (subject to the extent to which CUE declined) (Allison *et al.*, 2010).

4. Measurement of CUE

While quite an extensive number of studies have dealt with CUE, its controls and its potential responses to climate change, the results are not unequivocal. As has been pointed out above, this is at least partly caused by different methodologies used to measure CUE. Manzoni *et al.* (2012) identified as much as five fundamentally different approaches, based either on the uptake or the decrease of a substrate, the increase in microbial biomass, microbial growth rate, and finally a model-based approach. Of those methods, two have been established as standard methods: for terrestrial systems, a biomass-based method using stable isotope tracers; for aquatic systems, a growth-rate based method with radioactive tracers (Manzoni *et al.*, 2012).

   The standard method in terrestrial systems is based on the incubation of soil with a ^{13}C-labeled labile carbon substrate, most commonly glucose. To determine biomass increase, the amount of ^{13}C assimilated by microbial cells is measured by chloroform-fumigation extraction. Respiration, on the other hand, is measured by tracing ^{13}C into CO_2 and thus only encompasses respiration from the labeled substrate (Brant *et al.*, 2006; Sinsabaugh *et al.*, 2013). The corresponding CUE value that is obtained by this method is thus only based on the utilization of a single labile substrate. Natural substrates in soil, on the other hand, are a mixture of different compounds, including complex substrates, whose decomposition may require a large number of different enzymatic pathways and steps, and therefore come at higher energy cost (Frey *et al.*, 2001; Manzoni *et al.*, 2012). Moreover, the assimilation of a labeled substrate into biomass does not necessarily represent growth; for glucose, for example, it has been shown that it can be rapidly taken up by microbial cells for storage (Frey *et al.*, 2001; Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). Finally, the high input of a labile substrate associated with this method is not representative of environmental conditions. It may lead to shifts in the soil community by favoring microbes that are competitive at high substrate concentrations to the detriment of others that are adapted to low substrate concentrations (Schwartz *et al.*, 2007; Sinsabaugh *et al.*, 2013); the measured community CUE might thus mainly reflect the CUE of the first group (Sinsabaugh *et al.*,

2013). The high substrate input might also result in a decoupling of microbial catabolism and anabolism with a short-term stimulation of biomass production that is not in accord with the long-term energy budget of the cell (Sinsabaugh *et al.*, 2013). Taken together, all of these factors will likely result in an overestimation of CUE. Given these methodological limitations, it is not surprising that many studies measure CUE values in soil that are well above the thermodynamic maximum, and that the average CUE measured for soils (0.55) is higher than predicted on the basis of kinetic and stoichiometric constraints (Sinsabaugh *et al.*, 2013).

In aquatic systems, bacterial production is usually determined by the incorporation of ^{3}H-thymidine into DNA (Fuhrman & Azam, 1980) or ^{14}C-leucine into protein (Kirchman *et al.*, 1985). This approach, based on DNA and protein synthesis rather than biomass incorporation, is much better suited to reflect true growth (Sinsabaugh *et al.*, 2013). Although it relies on growth estimates based on a single substrate, respiration is not restricted to respiration from the labeled substrate but measured as total CO_2 production or O_2 consumption. As a result, the average CUE measured in aquatic systems is 0.26 and thus considerably lower than the average CUE measured in terrestrial systems (Sinsabaugh *et al.*, 2013). The thymidine and leucine methods have occasionally been applied to soils, where they yielded CUE values similar to those found in aquatic systems (Bååth, 1994; Tibbles & Harris, 1996; Rousk & Bååth, 2011). However, they were never generally established for terrestrial systems for several reasons. Firstly, the incubation with a radiolabeled tracer has to be performed in a soil slurry or suspension of microbial cells extracted from soil (Rousk & Bååth, 2011). This is very problematic for the determination of growth in soils, since soil structure is an important factor that controls microbial growth, for example by physical protection of soil organic matter or disconnection of decomposers and their substrates (Jones & Edwards, 1998; Schmidt *et al.*, 2011; Resat *et al.*, 2012). Furthermore, both the leucine and the thymidine method are quite specific for bacteria, and indeed not even all bacteria take up exogenous thymidine and leucine (Bååth, 1990; Buesing & Gessner, 2003; Pérez *et al.*, 2010). While a similar method, based on ^{14}C-acetate incorporation into ergosterol, exists for fungi (Newell & Fallon, 1991), none of the methods includes all groups of decomposers.

5. Study aim: a new method for soils

As pointed out above, the standard method to determine CUE in soils has several shortcomings that are likely to result in an overestimation of CUE, while the method commonly used in aquatic systems probably gives a more realistic estimate, but is not readily

applicable to soils. Yet it is crucial to gain further insight into soil microbial CUE both with regard to current CUE values in different terrestrial environments and to factors controlling CUE: only then can CUE be adequately represented in biogeochemical models that predict the extent and effects of global change.

The aim of this study was to develop a new method for soils that would avoid the bias of the ^{13}C-based method and give a more realistic estimate of CUE in the environment. Our approach involved the estimation of microbial growth by labeling soil with ^{18}O-enriched water and subsequently tracing the ^{18}O into microbial DNA. Previous studies had demonstrated that ^{18}O from water was incorporated into microbial DNA and that this incorporation could be used as a proxy for microbial growth (Schwartz, 2007, 2009; Aanderud & Lennon, 2011). On the basis of these findings, we developed a protocol adapted for the determination of CUE in soils. Our approach has several advantages compared to the standard ^{13}C-based method:

(i) the formation of DNA is a much better measure for growth than the short-term incorporation of a labile substrate (Sinsabaugh *et al.*, 2013);

(ii) water is taken up by all microorganisms, yet cannot be used as an energy, carbon or nutrient source (Schwartz, 2007);

(iii) water addition to soil is a naturally occurring phenomenon in the environment;

(iv) CUE is determined from the utilization of substrates naturally occurring in the soil instead of a single labile substrate.

For these reasons, we expected our method to yield CUE values that are considerably lower than those obtained by the ^{13}C-based method and that are more in line with theoretical considerations that predict an average CUE of about 0.3. To test this hypothesis, we performed both the new and the standard method (using ^{13}C-labeled glucose) in three separate experiments. The first experiment included soils from three terrestrial ecosystems (grassland, forest, and arable land) that were sampled in Vienna, Austria. For the second experiment, we collected four soils from a colline vegetation zone around Gumpenstein, Austria: a rendzic cambisol, a cambic leptosol, and two dystric cambisols. Finally, the methods were also compared within a soil profile comprising five soil horizons from an entic podsol sampled in Bayreuth, Germany.

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II. MANUSCRIPT

Abstract

Microbial carbon use efficiency (CUE), defined as the amount of carbon that is used for growth relative to the total carbon taken up, is an important parameter determining soil carbon processing and storage. As such, it is an integral part of state-of-the-art biogeochemical models that predict the effects of global change. Because of thermodynamic and environmental constraints, the average CUE in soils is expected to be around 0.3. However, the current standard method for terrestrial systems, which is based on incubation with a ^{13}C-labeled labile substrate and tracing ^{13}C into microbial biomass and CO_2, yields an average CUE of 0.55. This high value is thought to be due to methodological shortcomings that result in an overestimation of CUE. We here describe a new protocol for the determination of CUE in soils that estimates growth from the incorporation of ^{18}O from labeled water into DNA. Our approach has several advantages compared to the ^{13}C method: (a) it is based on the utilization of all natural substrates present in soil, not a single added substrate; (b) growth is measured from DNA formation instead of biomass incorporation; (c) water addition, unlike the addition of a labile carbon compound, is a naturally occurring phenomenon in terrestrial ecosystems. To test our novel protocol, we compared its results to the results of the ^{13}C-based method for seven different soils and a soil profile comprising five horizons. The average CUE determined by our method was 0.39 (± 0.13), while the standard method yielded an average CUE of 0.80 (±0.10). We conclude that our protocol is a viable alternative to the current standard method. It yields realistic results that are in line with theoretical considerations and will thus allow valuable insights into CUE in terrestrial ecosystems in a changing climate.

1. Introduction

Microbial carbon use efficiency (CUE) is a key parameter to understand the fate of dead organic matter in terrestrial and aquatic systems. When organic matter is taken up by microorganisms, it can either be respired to CO_2, used for the production of new biomass (growth), excreted in the form of metabolites or extracellular enzymes, and/or stored in the cell (Manzoni *et al.*, 2012). CUE, also termed growth efficiency or growth yield, is defined as the amount of organic carbon that is employed into new biomass relative to the total uptake of organic carbon (e.g. del Giorgio & Cole, 1998; Manzoni *et al.*, 2012). Since storage and carbon excretion are usually assumed to be negligible compared to growth and respiration (at least under aerobic conditions), CUE is often determined in a simplified way as:

$$CUE = \frac{C_{produced}}{C_{produced} + C_{respired}}$$

where $C_{produced}$ is carbon incorporated into newly formed microbial biomass and $C_{respired}$ is carbon respired in the form of CO_2 (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013).

When soil microorganisms take up plant-derived carbon and use it for the production of new biomass, it has the potential to become soil organic matter and to be stabilized in the soil for long time periods (Manzoni *et al.*, 2012). Accordingly, a high CUE favors soil carbon storage, which is one of the major mechanisms that remove CO_2 from the short-term carbon cycle (Lal, 2008). The extent to which soil carbon storage can buffer – or exacerbate – increasing atmospheric CO_2 concentrations ultimately depends on the response of microbial CUE to rising temperatures, changed nutrient regimes or precipitation patterns, and on its adaptability to changing environmental conditions (e.g. Allison *et al.*, 2010; Wieder *et al.*, 2013). Despite this essential role, the current knowledge about microbial CUE and its variability in the light of global change is relatively poor (Manzoni *et al.*, 2012). This is largely due to the fact that the current standard methods to estimate microbial CUE do not give a realistic picture of CUE in soils under natural conditions. In terrestrial systems, the standard method to measure CUE is based on incubating the soil with a ^{13}C-labeled labile carbon substrate, such as glucose, and tracing the ^{13}C into microbial biomass and CO_2 to determine the fraction of the substrate that is incorporated into new biomass (Brant *et al.*, 2006). This method has several severe shortcomings that have been discussed in the recent literature (Sinsabaugh *et al.*, 2013). Firstly, the short-term incorporation of a labile substrate is not a good measure for growth and tends to result in an overestimation of production (Manzoni *et al.*, 2012; Sinsabaugh *et al.*, 2013). When a large amount of a labile carbon compound is

added to the soil, it is rapidly taken up by microorganisms, but not necessarily used for growth, i.e. the production of new biomass (Frey *et al.*, 2001). Moreover, the ^{13}C method is based on the utilization of a specific added substrate instead of the complex mixture of substrates that naturally occurs in the soil (Sinsabaugh *et al.*, 2013). Uptake and metabolization, however, are specific for each substrate (Frey *et al.*, 2013), and the CUE of one substrate will not reflect CUE when multiple substrates are used. For instance, a labile substrate such as glucose can be assimilated directly, whereas organic matter in soils usually has to be depolymerized by extracellular enzymes before it can be taken up by microbial cells; its incorporation into biomass will therefore come at greater respiratory cost (Schwartz, 2007). Additionally, high substrate inputs can cause a short-term stimulation of microbial growth, resulting in an initially high CUE that decreases rapidly when catabolic and maintenance processes take over (Sinsabaugh *et al.*, 2013). Since the input of a labile carbon compound does not reflect natural conditions in soils, it can also result in a community shift from microorganisms adapted to low substrate concentrations to others that are able to take advantage of high substrate concentrations for fast growth (Schwartz *et al.*, 2007; Sinsabaugh *et al.*, 2013). The resulting CUE will thus mainly reflect the CUE of a particular group of decomposing organisms rather than the whole microbial community. In addition, not all microorganisms are able to take up all carbon-containing compounds to the same extent, which excludes certain groups of microorganisms from measurement (Sinsabaugh *et al.*, 2013). Finally, the ^{13}C-based method only takes into account respiration of the added substrate, but it is unlikely that all energetic demands (which include not only growth, but e.g. also maintenance and enzyme production) can be satisfied directly by respiration of the added substrate (Sinsabaugh *et al.*, 2013). All these factors likely lead to an overestimation of CUE, and the method has been referred to as a measure of "instantaneous substrate use efficiency" rather than CUE (Sinsabaugh *et al.*, 2013). Given these limitations, it is not surprising that the mean CUE obtained by the ^{13}C-based method is 0.55, and thus considerably higher than the average CUE between 0.2 and 0.3 that is predicted by thermodynamic and stoichiometric considerations and measured in aquatic systems (Sinsabaugh *et al.*, 2013). The standard method for the estimation of bacterial production in aquatic systems is based on the determination of DNA or protein synthesis from incorporation of radiolabeled thymidine or leucine, respectively (Fuhrman & Azam, 1980; Kirchman *et al.*, 1985); a conversion factor is then applied to estimate microbial production from newly formed DNA or protein. Although this approach also relies on the input of a labile compound, the thymidine-incorporation method is likely to give a more realistic estimate of bacterial production (and thus of CUE), because only actively growing cells produce new DNA (Sinsabaugh *et al.*, 2013). It is, however, not readily applicable to soils, since it requires

incubation with the radiolabeled tracer in a soil slurry or cell suspension (Rousk & Bååth, 2011) and thus disregards the fact that structure is an important factor for microbial growth in soils (Schmidt *et al.*, 2011). In addition, the thymidine- and leucine-incorporation methods are quite specific for bacteria, which is particularly problematic in soils, where fungi are an important part of the decomposer community (Bååth, 1990; Buesing & Gessner, 2003). Indeed, exogenous thymidine and leucine are not even taken up by all bacteria (Pérez *et al.*, 2010), which introduces an additional bias. A similar method using ^{14}C-acetate incorporation into ergosterol has been developed for fungi (Newell & Fallon, 1991), but none of these methods include all soil microorganisms to the same extent.

We here present a new method for the determination of CUE in soils that estimates microbial growth from the incorporation of ^{18}O from labeled water into DNA. In the last few years, water enriched in the stable isotope ^{18}O has been successfully used to label the DNA of growing microorganisms in soil in the context of classical stable-isotope probing (SIP) (Schwartz, 2007, 2009; Aanderud & Lennon, 2011). In a proof-of-concept paper, Schwartz (2007) illustrated that incubation of soil with ^{18}O-enriched water resulted in sufficient incorporation of ^{18}O into DNA for subsequent separation of heavy (^{18}O-enriched) and light DNA on a cesium chloride gradient (^{18}O-SIP). A subsequent study demonstrated that, upon addition of ^{18}O-enriched water to soil, the ^{18}O enrichment of extracted DNA was linearly related to the ^{18}O enrichment of the added water in the first 72 hours after incubation, indicating that ^{18}O incorporation into DNA can be used as a proxy for microbial growth (Blazewicz & Schwartz, 2011). Aanderud and Lennon (2011) confirmed that ^{18}O is incorporated into all structural components of DNA (albeit unevenly) and that this incorporation is stable insofar as no further isotopic exchange of ^{18}O with water occurs once it has been incorporated into DNA.

Based on these findings, we developed a protocol that combines the advantages of the ^{13}C-substrate method and the thymidine-incorporation method while circumventing most of their problems. It is similar to the thymidine method insofar as it estimates the production of new microbial biomass from the formation of DNA, which almost exclusively occurs when cells are dividing and is consequently a better measure for growth than the short-term incorporation of a labile carbon compound. Instead of incubation in a soil slurry or cell suspension, however, a small amount of labeled water is added directly to the soil, an event that regularly occurs in the environment. Water is taken up by all groups of decomposing microorganisms – that is, bacteria, fungi and archaea – and at the same time is not used as an energy or nutrient source (Schwartz, 2007). Last but not least, growth and respiration are not measured on the basis of one added substrate, but of all substrates that are naturally present in the soil. For these reasons, the setup of our protocol is much closer to natural

conditions in soils and it can be expected to yield more realistic CUE values than the current standard method based on ^{13}C.

1.1. Procedural comments

The general workflow of our protocol is depicted in **Figure 1**. Soil is incubated with ^{18}O-labeled water for a period of 24 hours, and soil respiration is measured as accumulation of CO_2 during this time. After the incubation, microbial DNA is extracted and the ^{18}O content of the DNA is determined by isotope ratio mass spectrometry (IRMS). The amount of newly formed DNA in the labeled samples is determined by calculating the excess ^{18}O in DNA relative to the natural abundance of ^{18}O. A conversion factor is applied to estimate the production of microbial carbon from newly formed DNA; this conversion factor is determined in a parallel experiment by measuring soil microbial biomass with the chloroform-fumigation extraction method and relating it to microbial DNA content. Finally, CUE is calculated as carbon used for growth relative to total carbon that was taken up, the latter approximated as the sum of carbon used for growth and respired carbon.

For reasons detailed below, the incubation experiments are performed with small amounts of soil, which requires careful preparation and pre-tests. The amount of soil used for the incubation has to be suitable for both DNA extraction and determination of soil respiration. Our protocol is optimized for soil DNA extraction with FastDNA™ SPIN Kit for Soil (MP Biomedicals), which allows for an absolute maximum of 500 mg of soil per replicate (or less in the case of low-density organic soils); if larger amounts of soil are used, the sample has to be split into aliquots for the DNA extraction. Yet the soil must produce a sufficient amount of CO_2 during the incubation period to allow a reliable determination of respiration. To a certain extent, this problem could be circumvented by using incubation vials smaller than the 50-mL vials described in our protocol. However, for technical reasons, we used 12-mL gas samples for the respiration measurement, and pre-tests showed that the gas sampling becomes erratic with vial volumes smaller than 50 mL at this sample size (data not shown). This is probably due to a small influx of ambient air caused by the reduction of pressure in the vial when the sample is drawn. In any case, the intrinsic heterogeneity of soil sets a lower limit for the sample size, as very small amounts of soil will not be representative.

As for every SIP experiment, labeling is a key step in our protocol. The incorporation of the labeled compound – and thus the required enrichment of the stable isotope in the label – depends on the incubation time, the conditions of the incubation, and the properties of the microbial community that is studied, in particular its growth rate and CUE (Neufeld *et al.*, 2007; Jehmlich *et al.*, 2010). For 24-hour incubations at 15°C, we recommend an enrichment of 20 atom percent (at%) of ^{18}O in the final soil solution. We tested the protocol on a range of

different soils, and 20 at% ^{18}O enrichment resulted in an ^{18}O signal in DNA with sufficiently high resolution for measurement in all cases (**Supplementary Table 1**). The uniform distribution of the label in the sample is equally important. We recommend adjusting the soil to an appropriate percentage of its water holding capacity to ensure comparable conditions for different soils and experiments. It should be tested beforehand whether the amount of labeled water that will be added is sufficient to distribute evenly in the soil.

When working with isotopically enriched substances, particular care has to be taken to avoid carry-over of the enriched isotope to unlabeled control samples. At every step of the protocol, unlabeled samples should be handled before labeled samples, and equipment that comes into contact with the enriched substance must be cleaned thoroughly before further use. The handling of H_2^{18}O requires additional precautions, because there is rapid exchange of ^{18}O between water in the liquid and in the gaseous phase. The same applies to CO_2 in air, where the exchange is mediated by the carbonate system (Hsieh *et al.*, 1998). ^{18}O-enriched water should therefore be kept in small-volume gastight vials and should be transferred from one vial to the next with gastight syringes. In addition, the vials used for the incubation should be dried in a drying oven prior to the incubation to remove any residual humidity.

1.2. DNA extraction

A crucial step in the development of the protocol was the selection of a suitable soil DNA extraction method. When extracting DNA from soil, there is a typical trade-off between DNA yield and DNA purity (Miller *et al.*, 1999). A method suitable for our protocol had to recover a sufficient amount of DNA for subsequent analysis by mass spectrometry. In addition, the DNA had to be recovered as completely as possible in order to avoid an underestimation of the soil DNA content as well as a bias towards microbial groups with more easily extractable DNA (Roose-Amsaleg *et al.*, 2001; Feinstein *et al.*, 2009). At the same time, the DNA extract had to be sufficiently clean of extraneous compounds that would either interfere with measurement on the mass spectrometer or could become enriched in ^{18}O during the incubation, such as proteins. The latter is particularly important because our method is based on the assumption that only DNA, and no other oxygen-containing substances in the extract, incorporates ^{18}O from labeled water.

Numerous protocols are available for the extraction of DNA from soil, but all follow the same basic procedure. First, there is a lysis step (mechanical, chemical, and/or enzymatic) to disrupt microbial cells and bring nucleic acids into solution. The removal of proteins, humic substances, and other co-extracted compounds is done by one or more purification steps. In the conventional method, DNA is then precipitated with ethanol or isopropanol and the DNA pellet is dissolved in water (Roose-Amsaleg *et al.*, 2001). Another technique to recover DNA

from solution is the introduction of a silica matrix); the DNA binds onto the matrix and is subsequently eluted with water. This method is employed by commercially available DNA extraction kits (Lloyd-Jones & Hunter, 2001; Sambrook & Russell, 2001; Mumy & Findlay, 2004; Chen *et al.*, 2015). To identify the most suitable extraction method for our protocol, we performed a series of extractions to compare DNA yield as well as purity from oxygen-containing substances of two different approaches: (1) cell lysis with chloroform and isoamyl alcohol followed by precipitation with ethanol (conventional method), and (2) extraction with two commercially available kits: FastDNA™ SPIN Kit for Soil (MP Biomedicals), and PowerSoil® DNA Isolation Kit (Mo Bio Laboratories) (**Supplementary Table 2**). The extracted DNA was quantified fluorometrically by staining with PicoGreen® (Life Technologies), a fluorescent dye specifically binding to double-stranded DNA *(ds*DNA*)*, and measurement on a microplate reader (Infinite® M200, Tecan) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The purity of the DNA was evaluated by calculating the absorption ratios 260/280 and 260/230 spectrophotometrically (Nanodrop ND-1000, Thermo Fisher). DNA extracts were subsequently dried and analyzed for total oxygen content on a Thermochemical Elemental Analyzer (Thermo Fisher) coupled via Conflo III open split system to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Fisher). The total oxygen content in the dried extract was compared with the oxygen content of the DNA as expected from quantification by fluorometric assay to obtain information about the presence of non-volatile oxygen-containing compounds other than DNA in the extract. An optimized version of the conventional method as well as the extraction with FastDNA™ SPIN Kit for Soil (MP Biomedicals) achieved comparable DNA yields that were adequate for our protocol. While the conventional method generally resulted in a lower amount of extraneous substances in the DNA extract than extractions with the two kits, the extraction with FastDNA™ SPIN Kit also yielded DNA extracts sufficiently pure for analysis by mass spectrometry (**Supplementary Table 2**). We chose the extraction with FastDNA™ SPIN Kit as the most appropriate method because it is faster and easier to handle than the conventional method, and it is the simplest way to ensure that the procedure is reproducible for all laboratories and users. Moreover, DNA extraction with FastDNA™ Kit is a well-established method that has been used successfully in many studies to recover DNA that is sufficiently pure for downstream molecular biology analyses (Ando *et al.*, 2005; Mincer *et al.*, 2005; Baelum *et al.*, 2006; Ascher *et al.*, 2009). We therefore assumed that it efficiently removes compounds such as proteins that could also incorporate [18]O. This is supported by Nanodrop analysis of the DNA extracts, which indicated only low amounts of protein (**Supplementary Table 2**). The results also indicated high amounts of humic substances and possibly compounds used in the extraction (e.g. buffers, ethanol) in the extract, but we

assumed that this is not problematic since none of these compounds is likely to incorporate ^{18}O during the incubation period.

It is certainly possible to use other methods of DNA extraction, but their suitability for this protocol should be tested beforehand. The method of choice has to include a purification step that efficiently removes substances that may also have incorporated excess ^{18}O, in particular proteins. Yet methods that are intended for the recovery of very clean DNA and include several purification steps might not be suitable, because every purification step inevitably results in a loss of DNA (Miller *et al.*, 1999; Roose-Amsaleg *et al.*, 2001). To a certain extent, our protocol accounts for DNA losses during extraction by using an individual conversion factor for each soil, but low DNA recovery might still be problematic (e.g. if it results in discrimination against certain groups of soil microorganisms).

1.3.   Conversion factor for the estimation of microbial carbon from DNA

Our method relies on a conversion factor F_{DNA} for the estimation of microbial-biomass carbon (C_{mic}) in a soil from its DNA content ($C_{mic} = F_{DNA} \times DNA$). In comparison to other methods for the determination of soil microbial biomass, DNA extraction is relatively fast and uncomplicated, and its suitability as a predictor for microbial carbon in soils has been examined in several studies (Marstorp *et al.*, 2000; Gong *et al.*, 2001; Blagodatskaya *et al.*, 2003; Agnelli *et al.*, 2004; Leckie *et al.*, 2004; Gangneux *et al.*, 2011; Anderson & Martens, 2013; Fornasier *et al.*, 2014). While most of these studies found a good correlation between extractable DNA and C_{mic}, they differed with respect to the value of the conversion factor. In a review of seven studies, Joergensen and Emmerling (2006) found conversion factors that ranged from 2.2 to 14.5. In two recent studies, Anderson and Martens (2013) obtained an F_{DNA} of 5.0 in a comprehensive assay of 44 field and forest soils, while Fornasier *et al.* (2014) found factors between 12.0 and 63.5 (average 26.9, own calculation obtained from linear regression between DNA and C_{mic}) in eight mineral soils with very different properties. There are indications in the literature that the predictability of C_{mic} from DNA is somewhat poorer in high-biomass soils or, at least, that their F_{DNA} is higher than that of other soils (Leckie *et al.*, 2004; Anderson & Martens, 2013). Anderson and Martens (2013) attributed this to a decreased efficiency of the DNA extraction in soils with high biomass and excluded soils with more than 1500 µg C_{mic} g^{-1} soil from their analysis. Another explanation might be that soils with a very high biomass are often from organic layers, e.g. forest humus, in which fungi represent a dominant fraction of the biomass, and their ratio of carbon to DNA is higher and generally more variable than that of bacteria (Leckie *et al.*, 2004).

The current state of knowledge thus suggests that it is not possible choose a factor F_{DNA} from the literature for our protocol. Indeed, the large discrepancy in the literature regarding

the value of F_{DNA} is not surprising because different studies use different methods of DNA extraction, and the recovery of DNA from soils varies greatly between different methods (Miller *et al.*, 1999; Martin-Laurent *et al.*, 2001). This is also true for the estimation of microbial carbon: though most studies use chloroform-fumigation extraction (Marstorp & Witter, 1999; Gong *et al.*, 2001; Agnelli *et al.*, 2004; Leckie *et al.*, 2004), some also use the substrate-induced respiration technique (Blagodatskaya *et al.*, 2003; Anderson & Martens, 2013) or phospholipid fatty acid analysis (Widmer *et al.*, 2001). It follows that, assuming that a universal factor can be applied to all types of soils, it might still have to be established for each method of DNA extraction (and biomass estimation) separately. This is particularly true for our protocol, because most of the cited papers optimized the DNA extraction with a view to making the recovery as complete as possible and often did not include a purification step after cell lysis (Anderson & Martens, 2013; Fornasier *et al.*, 2014), whereas we had to ensure sufficient purity of the DNA from interfering compounds (see above).

To evaluate whether a common conversion factor F_{DNA} could be established for the purpose of our protocol, we determined the correlation between *ds*DNA extracted with FastDNA™ SPIN Kit for Soil and C_{mic} estimated by chloroform-fumigation extraction in a variety of soils. In addition, we tested whether the amount of soil used for the extraction had a substantial influence on the conversion factor. After extraction with FastDNA™ SPIN Kit (MP Biomedicals), soil DNA was quantified by staining with PicoGreen® fluorescent dye (Life Technologies) and measurement on a microplate reader (excitation 480 nm, emission 520 nm; measurement in three replicates). C_{mic} was determined by the chloroform-fumigation extraction method as described in Vance *et al.* (1987). Five g of 2-mm sieved fresh soil were extracted with 0.5 M K_2SO_4 for 30 minutes in two batches: one batch was extracted directly, one batch after 48-hour fumigation with chloroform. Dissolved organic carbon was measured in both extracts with a TOC Analyzer (TOC-V CPH E200V, Shimadzu) and microbial-biomass carbon was calculated as the difference between fumigated and non-fumigated samples, corrected for extraction efficiency (kEC = 0.45). We conducted two separate experiments that resulted in very different average F_{DNA} values. An extraction series of 17 soils (four from fields and arable land, seven from forests, six from grassland sites) yielded an F_{DNA} of 49.1 ($r^2 = 0.88$, p < 0.001) (**Figure 2**). The soils used in this experiment generally had high C_{mic} contents (ten soils with over 1500 µg C_{mic} g^{-1} soil dry weight [dw]) and we used 500 mg of fresh soil for the DNA extraction. In a second experiment, we determined F_{DNA} on the basis of 16 soil samples with low C_{mic} contents (14 below 1500 µg g^{-1} soil dw): three soil profiles à four horizons from a luvic stagnosol, entic podsol, and podsolic stagnosol, two organic layers (from the entic podsol and the podsolic stagnosol profile), and two soils from arable land. The amount of soil used for DNA extraction was adapted to soil density and

expected C_{mic} (and DNA) content: 200 mg of soil were used for low-density soils with an expected high C_{mic} content (organic layers, soil depths 0-7 cm, arable soils), and 500 mg for high-density soils with expected lower C_{mic} content (soil depths below 7 cm). The conversion factor obtained from the linear regression was 10.9 ($r^2 = 0.92$, $p < 0.001$) (**Figure 3**). The two O horizons (with over 1500 µg C_{mic} g^{-1} soil dw) were identified as outliers (boxplot performed in R) and omitted from the analysis. The F_{DNA} values determined by the two separate experiments thus varied by a factor of almost five. The lower conversion factor found in the second experiment may be attributable to both the lower C_{mic} content of the tested soils and to the fact that the amount of soil used in the extraction was adapted to soil density and expected C_{mic} and DNA content. Both effects are shown in **Figure 4** (data provided by Marie Spohn, unpublished), where DNA extraction efficiency is displayed as a function of the amount of soil used in the extraction. The extraction efficiency decreases (and, as a consequence, F_{DNA} increases) with increasing amounts of soil. This is true for soils with high and low C_{mic} contents, but the decrease in efficiency is more pronounced in soils that are high in C_{mic}.

We conclude that, on the basis of our current data set, the establishment of a common F_{DNA} across all soil types is not feasible, as the extraction effiency depends both on soil properties and the amount of extracted soil. While two separate experiments show good correlations between extractable dsDNA and C_{mic}, the F_{DNA} obtained from these experiments varies by a factor of 5, and the factors calculated for single soils vary between 2.8 and 66.3. We therefore suggest that the relationship between extractable dsDNA and C_{mic} is established for each soil, so that the increase in C_{mic} from DNA can be calculated with a soil-specific F_{DNA}. Accordingly, our protocol includes quantification of DNA in the soil samples and a separate experiment to estimate C_{mic} in the soil by the chloroform-fumigation extraction method.

1.4.   Limitations of the method

Although our method has many advantages and simulates environmental conditions more closely than other methods for the determination of CUE in soils, it is important to keep in mind that the experiment as described below is still a microcosm experiment with all its limitations. The samples are removed from their natural environment and homogenized, which will disrupt larger soil aggregates as well as fungal hyphae. Our protocol also requires the amount of soil used per sample replicate to be rather small, which might be problematic with a view to the high heterogeneity of soil with regard to both microbial community and abiotic parameters. This could, however, be overcome by extracting several aliquots of a sample and then combining the DNA for further analysis. Furthermore, while there is no

artificial substrate input that directly selects for certain microorganisms, the addition of water to a fairly dry soil can still result in a shift of the microbial community (Schwartz, 2007), which was confirmed by Aanderud and Lennon (2011) in a study using DNA-SIP with $H_2^{18}O$. It should be pointed out that it is possible to adapt the protocol to larger soil samples, such as small soil cores, which circumvents most of these problems. This will however require larger amounts of labeled water, rendering the experiment substantially more expensive.

Another point to note is that, as with the thymidine-incorporation method, the estimation of total microbial growth depends to quite a large extent on the relative purity of extracted soil DNA and on the conversion factor that is applied to calculate the increase of C_{mic} from DNA. Our calculation of newly formed DNA is based on the assumption that the enrichment of ^{18}O in the DNA extract derives solely from DNA. In our preliminary results, we saw no indication that this assumption is violated for DNA extractions with FastDNA™ SPIN Kit for Soil (MP Biomedicals). Nanodrop analysis indicates that the DNA extract does not contain large amounts of protein, and while the content of humic substances and chemicals used in the extraction appears to be quite high, it is unlikely that these compounds will incorporate significant amounts of ^{18}O (**Supplementary Table 2**). In addition, the factor that is applied for the estimation of microbial-biomass carbon from DNA has a large influence on the calculated CUE, and is poorly constrained. Our results indicate that the application of a common factor across all soil types is not possible, which makes it necessary to establish a separate factor for each soil. Using a specific conversion factor also avoids a potential bias due to high abundances of microorganisms with large amounts of plasmid DNA that might prevail in some soils. If a common factor was used, microbial growth in such soils would be overestimated in relation to other soils. However, if F_{DNA} is determined separately, with biomass being determined under conditions that are equal to the conditions of the incubation (e.g. with regard to soil moisture, water addition and temperature), this will result in a lower F_{DNA} for such soils, reflecting a relation between DNA and biomass that can be applied to the incubation experiment.

In addition to water in the soil, internal cell water will also be used for microbial growth processes. If internal water was used preferentially or the exchange between soil water and internal cell water was slow, this would be problematic for our method, because not all newly formed DNA would incorporate ^{18}O. While this is very difficult to clarify, we assume that it is not a problem for our protocol. There is constant exchange of H_2O between the inside and outside of cells, and an incubation time of 24 hours should be sufficient to ensure that ^{18}O equilibrates between the inside and outside of cells.

Finally, we cannot rule out that there is an isotopic effect of ^{18}O on microbial growth and/or respiration. A recent study showed that *Escherichia coli* cultures grown in ^{15}N media

had a decreased growth rate compared to cultures grown in unlabeled (^{14}N) media; that is, apparently a mass difference as small as $^1/_{14}$ can have an effect on growth (Filiou *et al.*, 2012). On the other hand, no significant decrease in growth rates has been observed for bacteria subjected to up to 50 at% deuterium-enriched water in their media (Berry *et al.*, 2015). It is not known whether an isotopic effect exists for ^{18}O-labeled water and if so, whether it applies to growth and/or respiration. If growth and respiration are affected to the same extent, it would not be problematic since CUE is a ratio.

1.5. Application of the method

In preliminary experiments, the ^{18}O-based method was used successfully with soils of different composition. The results were compared with CUE estimated with the ^{13}C-based method adapted from Schindlbacher *et al.* (2015). Four different mineral soils were examined: a rendzic cambisol, a cambic leptosol, and two dystric cambisols. All soils were incubated at 15°C in the dark for 24 hours with ^{18}O-enriched water or ^{13}C-enriched glucose, respectively. The [18]O method was performed as described in the protocol. For the ^{13}C method, 2 g of 2-mm sieved soil were mixed with ^{13}C-labeled glucose solution (20 µg glucose g^{-1} soil fresh weight) and incubated in 100-mL Schott bottles with butyl rubber plugs. Gas samples were taken at the start and end of the incubation, and CO_2 concentration and at% [13]C in CO_2 were measured on a Gas Bench II (Thermo Fisher) coupled to a Delta V Advantage IRMS (Thermo Fisher). Respiration from substrate was determined from the increment in labeled CO_2 during the incubation period. After the incubation, soil samples were split into two equal parts and microbial biomass was determined by chloroform-fumigation extraction. The at% [13]C in the non-fumigated and fumigated soil extracts was measured by injecting samples with an HPLC autosampler into a Finnigan LC-Isolink Interface (Thermo Fisher) coupled to a Delta V Advantage IRMS (Thermo Fisher). Microbial growth from the labeled substrate was determined by comparing ^{13}C in the non-fumigated and fumigated samples as described by Schindlbacher *et al.* (2015).

As shown in **Table 1**, microbial production as obtained by the [18]O method varied by a factor of up to 10 between the different soils, whereas the values obtained ^{13}C method varied only by a factor of 3, similar to respiration. In line with our expectations, the CUE determined by [18]O method was significantly lower than the CUE determined by ^{13}C method for all soils. CUE ranged from 0.34 to 0.49 for the ^{18}O-based and from 0.81 to 0.94 for the ^{13}C-based method (**Figure 5**). To ascertain whether different incubation times would yield similar CUE values, we repeated the experiment with a grassland soil and incubated it separately for 24 hours and for 48 hours. The calculated CUE was slightly lower in the 48-hour incubations for both methods, but the difference was not statistically significant (**Table 2**), demonstrating that

our method was not sensitive to variations in incubation time, as has been suggested for other methods (see also Hagerty *et al.*, 2014).

In addition, we compared the novel ^{18}O-based method to the classical ^{13}C-based method in different soil horizons (**Figure 6**). Both methods were performed in parallel on five samples from an entic podsol soil profile: O horizon, Ahe horizon (0-3 cm), Ae horizon (3-7 and 10-15 cm, respectively), and Bw horizon (34-40 cm). As expected, both methods indicated that growth and respiration generally decreased with increasing soil depth (**Table 3**), although differences in the lower soil depths were not significant. CUE determined by ^{13}C method varied between 0.90 in the O horizon and 0.59 in the Bw horizon and declined with increasing soil depth (**Figure 6**). The pattern estimated by the ^{18}O method was very different: despite substantial differences in activities, it was high in the upper soil layers at 0.62, declined until the Ae horizon (depth 10-15 cm) to 0.16 and then increased again almost to the level of the O horizon. Although both growth and respiration varied by a factor of 100 between O horizon and Bw horizon (at 34-40 cm depth), there was no significant difference between CUE in both horizons (**Figure 6**). This may be due to several environmental parameters that change within a soil profile and have opposing effects on CUE. Upper soils typically contain a larger portion of organic matter that is biologically easily available and (on account of its chemical composition or degree of reduction) energetically less costly to incorporate into biomass, resulting in a comparatively high CUE in upper soils (Manzoni *et al.*, 2012). On the other hand, both total carbon availability and the ratio of carbon to nutrients (in particular nitrogen) decrease with soil depth, which should increase microbial CUE as organisms strive to use the available carbon as efficiently as possible and nutrient limitation declines (Manzoni *et al.*, 2012). The observed increase in CUE in the two lowest soil depths could be explained by both of these factors coming into effect as the chemical quality of the substrate becomes less and less important.

1.6. Concluding remarks

Our protocol describes a new approach to estimate the CUE of soil microbial communities that encompasses all groups of microorganisms. Labeling with ^{18}O-enriched water will allow a better insight into CUE under environmental conditions than previously described methods for soils, as it is not based on an added labile substrate but on substrates that naturally occur in the soil. Moreover, our estimation of microbial production derives from the amount of newly formed DNA and is consequently a measure of true growth. In addition, the novel method allows the determination of microbial turnover times of DNA (as production of new DNA over the amount of DNA present in the sample) and could be used to link CUE to the active microbial community by ^{18}O-DNA-SIP. Our protocol has been used successfully on different

kinds of soil. The average CUE obtained by our method across different soil types was 0.39 (± 0.13) compared to an average CUE of 0.80 (± 0.10) with the ^{13}C-based method, which is well in line with theoretical considerations about CUE in soils.

2. Materials

REAGENTS

- FastDNA™ SPIN Kit for Soil (MP Biomedicals *116560200*) **! CAUTION** Kit contains harmful substances. Handle using appropriate safety equipment.
- Molecular biology grade water (e.g. 5 Prime *2500010*)
- ^{18}O-enriched water ≥ 97 at% (e.g. CortecNet *139803B-P*, Campro Scientific *CS03-388_103*)
- Liquid N_2 **! CAUTION** Harmful. Handle using appropriate safety equipment.
- Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies *P7589*) **! CAUTION** Kit contains harmful substances. Handle using appropriate safety equipment.
- Chemicals for calibration of TC/EA-IRMS: Glutamic acid (p.a. grade), Potassium nitrate (p.a. grade), USGS-34 (Potassium nitrate), USGS-35 (Sodium nitrate), IAEA-601 (Benzoic acid), IAEA-602 (Benzoic acid)
- Chemicals for calibration of Gas Bench-IRMS: Reference gas (CO_2, Air Liquide), CO_2 standards with known isotopic composition (ISOTOP, Sigma Aldrich)
- Chloroform, amylene-stabilized (Sigma Aldrich *34854*) **! CAUTION** Harmful. Handle using appropriate safety equipment.
- KCl (p.a. grade)
- Air with known CO_2 concentration

EQUIPMENT

- Reaction tubes, 2 mL, screw cap (Greiner Bio-One *722201*)
- Reaction tubes, 2 mL, snap cap (Eppendorf *0030120.094*)
- Pipettes for various volume ranges
- Pipette tips for various volume ranges
- Headspace vials, 50 mL, crimp neck (Supelco *SU860134*)
- Aluminum crimp caps, diameter 20 mm, open center (Supelco *508500*)
- 250-mL glass beaker
- Water jet pump

- Aluminum dishes (Rotilabo® sample bowls, Roth *5494.1*)
- Gastight vials, 1.5 mL, with rubber septum (e.g. Bruckner Analysentechnik *610002* & *611003*)
- Crimper for 20-mm caps
- 2-mm mesh screen
- Silica gel
- 30-mL syringes with Luer adapter (B. Braun *4616308F*)
- Luer lock with valve
- Needles (B.Braun *4657527*)
- Exetainers®, 12 mL, flat bottom, soda glass, with screw cap and septum (Labco *738W*)
- Silver capsules for liquids, 3.5 x 4.0 mm, Ag 99.99 (IVA Analysentechnik *84.9912.36*)
- Drying oven
- Desiccator
- Semi-micro analytical balance (d = 0.01 mg)
- Micro-analytical balance (d = 0.0001 mg)
- Incubator
- Centrifuge with reactor for 2-mL reaction tubes (for 14,000 x g)
- Gastight syringe, 100 µL (Hamilton *SYR 1710 RN*) with removable needle, small hub, 22s gauge, point style 5, 2.7 inches (Hamilton *RN 7731-03*)
- Gasbench II (Thermo Fisher) coupled to a Delta V Advantage IRMS (Thermo Fisher)
- Thermochemical Elemental Analyzer (TC/EA) (Thermo Fisher) coupled via Conflo III open split system to a Delta V Advantage IRMS (Thermo Fisher)
- TOC Analyzer (TOC-V CPH *E200V*, Shimadzu)
- Microplates, 96 wells, F-bottom, black (Greiner Bio-One *655076*)
- Microplate reader (Infinite® M200, Tecan)
- Chemical-resistant vacuum pump
- 20-mL scintillation vials with screw caps (Sarstedt *73.662.500*)
- Isoprene rubber stoppers, diameter 20 mm (Supelco *27234*)
- Orbital shaker

- Ash-free cellulose filters (Sigma Aldrich *Z241180*)
- Funnel with filter (or 30 mL-syringe with filter cotton)
- Equipment for gas exchange (gas line and/or gasbag)

EQUIPMENT SETUP

50-mL headspace vials & 1.5-mL gastight vials Dry the vials in a drying oven at 60°C for a minimum of 6 hours before use to remove residual moisture.

3. Procedure

Sampling and preparation of soil

1| Collect a minimum of 200 g of field-most soil. About 100 g of 2 mm-sieved soil will be required for the protocol.

2| Pass the soil through a 2-mm mesh screen to remove stones and large roots.
■ **PAUSE POINT** The fresh soil should be processed as soon as possible. If necessary, it can be stored at 4°C in the dark for several days.

3| Three to seven days prior to the incubation, pre-incubate the soil in the dark at the temperature chosen for the experiment. Moisture loss and anoxic conditions should be avoided.
▲ **CRITICAL STEP** Pre-incubation at the incubation temperature is advisable to avoid effects of different soil temperatures prior to the incubation.

Preparation of incubation

4| One day prior to the incubation, weigh three replicates of 5 g of soil into an aluminum dish and dry in a drying oven at 105°C for 12 to 24 hours.

5| Determine the amount of soil that will be used for the incubation. Typically, 500 mg will be used for mineral soils and 200 mg for organic soils.
<u>Soil weight **x** = soil per replicate [mg]</u>
▲ **CRITICAL STEP** Excessive headspace in the incubation vial should be avoided to prevent the development of a CO_2 gradient in the vial.

6| After 12 to 24 hours, take the aluminum dishes (step 4) out of the drying oven and place them in a desiccator with silica gel for a minimum of 30 minutes.

7| Weigh the dried soil and calculate the water content of **x** mg of fresh soil. This value (*approximate* water content) is used for the preparation of the labeling solution (step 11).
<u>Water content **W** = water in **x** mg of fresh soil [μL]</u>

8| On the day that the incubation is started, repeat step 4 with five replicates of 5 g of soil.

9| After 12 to 24 hours, repeat steps 6 and 7 with the samples generated in step 8. The water content determined with these samples (*accurate* water content) is used for the gravimetric calculation of ^{18}O enrichment in soil water.

10| Determine the amount of water to be added for the incubation. This is best done by determining the water holding capacity of the soil and calculating the amount of water that has to be added to adjust the soil to an appropriate percentage of its water holding capacity (e.g. 60%).
(i) Prepare a funnel with filter or a 30-mL syringe with filter cotton. If using filter cotton, wet the filter cotton beforehand.
(ii) Place approximately 20 mL of soil in the funnel or syringe. Note the weight of the soil as well as the tare weight of funnel, filter and soil (or syringe, filter cotton and soil).
(iii) Fill the funnel (syringe) with water repeatedly until the soil is saturated with water, then leave it to drain for a minimum of one hour.
(iv) After a minimum of one hour, weigh the funnel (syringe) with soil.
(v) Calculate the water holding capacity of the soil as percentage of water left in the soil after draining per weight dry soil.
(vi) Calculate the amount of water required to adjust **x** mg of soil to an appropriate percentage of its water holding capacity (e.g. 60%).
Water addition **A** = water to be added to **x** mg soil [µL]
▲ **CRITICAL STEP** Keep in mind that the amount of water has to be sufficient to ensure even distribution of the label in the soil sample.

Labeling and incubation start

11| On the day of the incubation start, prepare a dilution of ^{18}O-enriched water ($\approx$ 99 at%) with non-enriched water (molecular biology grade) in a gastight vial using gastight syringes. The aim is to achieve an enrichment of 20 at% ^{18}O in the final soil water, i.e. the sum of labeling solution and soil water. The required at% ^{18}O of the labeling solution (at%$_{added}$) is calculated according to the following formula:

$$\text{at\%}_{added} = \frac{20 \times (W + A) - 0.2 \times W}{A}$$

where **A** is the water to be added to **x** mg soil [µL] and **W** is the approximate water content of **x** mg soil [µL]. 20 is the target at% ^{18}O in the final soil water and 0.2 is the average natural abundance at% ^{18}O.
▲ **CRITICAL STEP** Keep ^{18}O-enriched water and its dilutions in small-volume gastight vials (e.g. 1.5-mL gastight vials with rubber septum) and use gastight syringes (e.g. Hamilton

syringes) to transfer water from one vial to the next to minimize loss of ^{18}O due to evaporation and isotopic exchange.

12| Place **x** mg of soil into a 2-mL reaction tube (step 5). Prepare two sets of samples (minimum three replicates each): one for incubation with ^{18}O-enriched water (labeled samples) and one for the determination of natural abundance of ^{18}O (natural abundance samples). Place each 2-mL reaction tube into a 50-mL vial with crimp neck (incubation vial). In addition, prepare eight incubation vials with empty 2-mL reaction tubes (CO_2-start vials).

13| Close the incubation vials with soil samples as well as the CO_2-start vials with rubber stopper and aluminum cap and crimp. Using a gas line and/or gasbag, exchange the air in all vials (soil and no soil) with air of a known CO_2 concentration by evacuating for 30 seconds and flushing 3 to 4 times. Avoid excessive evacuation to prevent moisture loss from the soil. Note the time (start point for CO_2 measurement).
▲ **CRITICAL STEP** Take care to treat the CO_2-start vials in the same way as the incubation vials. If several vials are evacuated and flushed at once, be sure to include CO_2-start vials in every batch.

14| Apply **A** µL of molecular biology grade water to half of the soil samples (natural abundance samples) by inserting a gastight syringe through the rubber stopper; then apply **A** µL of the labeling solution to the other half of the samples (labeled samples). Note the time for each sample (start of ^{18}O incorporation into DNA).
▲ **CRITICAL STEP** Test the application of water to the soil sample through the vial septum beforehand. If necessary, use a longer needle.

15| Incubate all samples for 24 to 48 hours in the dark at the intended incubation temperature.

Incubation stop

16| At the end of the incubation period, take gas samples (at least 13 mL per replicate) from the CO_2-start samples and the labeled samples with a 30-mL syringe with Luer lock and valve and transfer them into pre-evacuated 12-mL Exetainers®. Note the time for the labeled samples (end point for CO_2 measurement). Gas samples from the natural abundance samples are not required.
■ **PAUSE POINT** Gas samples in Exetainers® can be stored in a desiccator with silica gel for several days before analysis.

17| After taking the gas samples, open the natural abundance samples, take out the 2-mL tube with soil with tweezers and close with screw cap. Flash-freeze in liquid N_2 and note the

time for each sample (stop of ^{18}O incorporation into DNA). Proceed in the same way with the labeled samples.

! CAUTION Liquid N$_2$ is harmful. Handle using appropriate safety equipment.

▲ CRITICAL STEP Close each vial immediately to avoid isotopic exchange of ^{18}O between soil water and air.

■ PAUSE POINT Soil samples can be stored at -20°C for several days or at -80°C for several weeks before DNA extraction.

DNA extraction and quantification

18| Transfer the frozen soil samples to a Lysing Matrix E Tube (included in FastDNA™ SPIN Kit for Soil, MP Biomedicals). According to the manufacturer, 250 to 500 µL of empty space should be left in the tube.

? TROUBLESHOOTING The soil sample is too voluminous to fit into the Lysing Matrix E Tube. Solution: Partition the sample into two aliquots.

19| Process the soil samples with FastDNA™ SPIN Kit for Soil (MP Biomedicals) as indicated by the manufacturer with the following modifications:

- Extend the centrifugation time after bead-beating to 15 minutes (Manual for FastDNA™ SPIN Kit, step 5).
- Do not discard part of the binding matrix suspension but transfer the whole suspension onto the SPIN™ filter in three steps à 600 µL (Manual, step 11).
- For the final elution step, use 100 µL of molecular biology grade water instead of DNAse/Pyrogen-free water (DES) provided in the kit (Manual, step 16) to reduce the amount of extraneous substances in the DNA extract.

Note the exact weight of the DNA extract by weighing the catch tube without and with the extract.

! CAUTION The kit contains harmful substances. Handle using appropriate safety equipment.

■ PAUSE POINT The DNA extracts can be stored at -20°C for several days or at -80°C for several weeks.

20| Quantify the DNA in the extract by fluorescence measurement after staining with a DNA-specific fluorescent stain such as Quant-iT™ PicoGreen® dsDNA Reagent (Life Technologies). At typical DNA concentrations, it will be sufficient to use a 200-fold to 300-fold dilution of the DNA extract (this requires 1 to 2 µL of the extract). Prepare a standard series with lambda DNA on the same plate as the samples. Measure fluorescence on a microplate reader (excitation at 480 nm, emission at 520 nm, optimal gain) and express DNA as µg DNA g^{-1} soil dry weight.

▲ CRITICAL STEP Measure the standards and the samples at least in triplicates.

! CAUTION PicoGreen® binds to nucleic acids and should be treated as a potential mutagen. Handle with care and use appropriate safety equipment.

Analysis

21| Measure the CO_2 concentration in CO_2-start samples (start concentration) as well as the labeled samples (end concentration) on a Gasbench-IRMS (step 16). Express respiration during the incubation period as CO_2-C [ng h^{-1} g^{-1} soil dry weight].

22| Dry the complete DNA extract (typically 60 to 90 µL per sample) in silver capsules for liquids fit for analysis on a TC/EA-IRMS. If applicable, pool DNA extracts of the same sample. If the silver capsules cannot hold the total DNA extract, transfer the maximum amount, then dry in a drying oven at a maximum temperature of 60°C. Repeat until the whole DNA extract is dried.

23| Fold the silver capsules containing the extracted soil DNA.

24| Determine the at% ^{18}O and the total amount of oxygen on a TC/EA-IRMS.

Chloroform-fumigation extraction

25| Determine microbial-biomass carbon in the soil by chloroform-fumigation extraction (CFE) as described in Vance *et al.* (1987).
▲ CRITICAL STEP The soil samples used for the chloroform-fumigation extraction should be treated in the same way as the incubation samples (e.g. with regard to water addition, acclimation to a certain temperature) to avoid a bias due to different conditions.
■ PAUSE POINT The soil extracts obtained by CFE can be stored at -20°C for several weeks.

26| Analyze the extracts (if necessary, in diluted form) on a TOC Analyzer. Express the content of microbial-biomass carbon in the sample as µg C_{mic} g^{-1} soil dry weight.

● TIMING OF THE INCUBATION

Note: Timing data for 40 samples (maximum number of parallel samples recommended)

1 week before the incubation

- Collect and prepare soil (steps 1-3)

1 day before the incubation

- Water content (step 4): 1 h
- *Optionally* - prepare incubation (steps 5 & 10): 2-3 h

Incubation day 1

- *If not done on previous day* - prepare incubation (steps 5 & 10): 2-3 h
- Water content (steps 6-8): 1 h
- Labeling and incubation start (steps 11-15): 3 h

Incubation day 2

- Water content (step 9): 1 h
- Incubation stop (steps 16-17): 3 h

● TIMING OF ADDITIONAL STEPS

Note: Timing data for 24 samples (maximum number of parallel samples recommended)

- DNA extraction (steps 18-19): 2-3 h
- DNA quantification (step 20): 3-4 h
- Analysis of gas samples (step 21): 20 h per run (handling 15 min)
- Preparation of DNA extract for analysis (steps 22-23): 1 h
- Analysis of DNA samples (step 24): 22 h per run (handling 15 min)
- Chloroform-fumigation extraction (step 25): 2 h on day 1, 4 h on day 2
- Analysis of CFE samples (step 26): 16 h per run (handling 1 h)

4. Anticipated results

4.1. Calculation of DNA formation

Measurement of the dried DNA extract on TC/EA-IRMS yields the total oxygen (O) content as well as the at% ^{18}O of non-volatile compounds in the DNA extract. In incubation experiments with 20 at% enrichment of ^{18}O in the final soil water, we obtained a mean O content of 132 (±7) μg with a mean at% ^{18}O of 0.211 (± 0.0045). We observed a total amount of O that was about 20 to 50 times higher than expected from the DNA content, which is in all probability due to oxygen-containing extraction reagents and co-extracted humic substances. We do not take this into account in our calculations, as we assume that these "contaminating" substances do not incorporate ^{18}O during the incubation, and unlabeled natural abundance samples are used to correct for the ^{18}O at% in the O blank.

We aimed for an enrichment of approximately 20 at% ^{18}O in the final soil solution. This proved to result in sufficient enrichment in DNA while at the same time limiting the required volume of (expensive) ^{18}O-enriched water. The exact enrichment of ^{18}O in the final soil solution (at%$_{label}$) is obtained by the following formula:

$$\text{at\%}_{label} = \frac{\text{at\%}_{added} \times A + 0.2 \times W}{W + A}$$

where at%$_{added}$ and A refer to the at% and amount of the labeled water respectively (Procedure, steps 10 and 11), and W is the accurate soil water content (Procedure, step 9). 0.2 is the average natural abundance at% ^{18}O (McConville et $al.$, 1999).

The amount of DNA that was produced during the incubation period (DNA$_{produced}$) is calculated as follows:

$$\text{DNA}_{produced} = \text{O}_{total} \times \frac{\text{at\%}_{excess}}{100} \times \frac{100}{\text{at\%}_{label}} \times \frac{100}{31.21}$$

where O$_{total}$ is the total O content of the dried DNA extract, at%$_{excess}$ is the at% excess ^{18}O (surplus at% ^{18}O compared to the mean at% ^{18}O of natural abundance samples), and at%$_{label}$ is the at% ^{18}O in the final soil solution. 31.21 refers to the weight% of O in DNA according to an average formula of DNA ($C_{39}H_{44}O_{24}N_{15}P_4$). We observed a mean excess of ^{18}O in DNA in the labeled samples of 0.007 (± 0.0042) at% (**Supplementary Table 1**).

4.2.   Estimation of microbial growth

To obtain the conversion factor F_{DNA} for the estimation of microbial-biomass carbon (C_{mic}) from DNA, the mean C_{mic} content [µg C_{mic} g^{-1} soil dw] (Procedure, step 26) is divided by the mean dsDNA content [µg DNA g^{-1} soil dw] (Procedure, step 20). The amount of biomass carbon produced during the incubation period ($C_{produced}$) is calculated as:

$$C_{produced} = F_{DNA} \times DNA_{produced}$$

where F_{DNA} is the conversion factor (ratio between C_{mic} and DNA) and $DNA_{produced}$ is the newly formed DNA. In our experiments comprising a total of twelve soil samples, F_{DNA} values ranged from 6.1 to 47.2 (**Supplementary Table 3**).

4.3.   Calculation of CUE

Both $C_{produced}$ and $C_{respired}$ are expressed as ng C h^{-1} g^{-1} soil dry weight and related according to the following formula to obtain CUE:

$$CUE = \frac{C_{produced}}{C_{produced} + C_{respired}}$$

Testing our protocol on twelve different soil samples, we obtained CUE values between 0.16 and 0.62 with an average of 0.39 (± 0.13) (**Supplementary Table 3**).
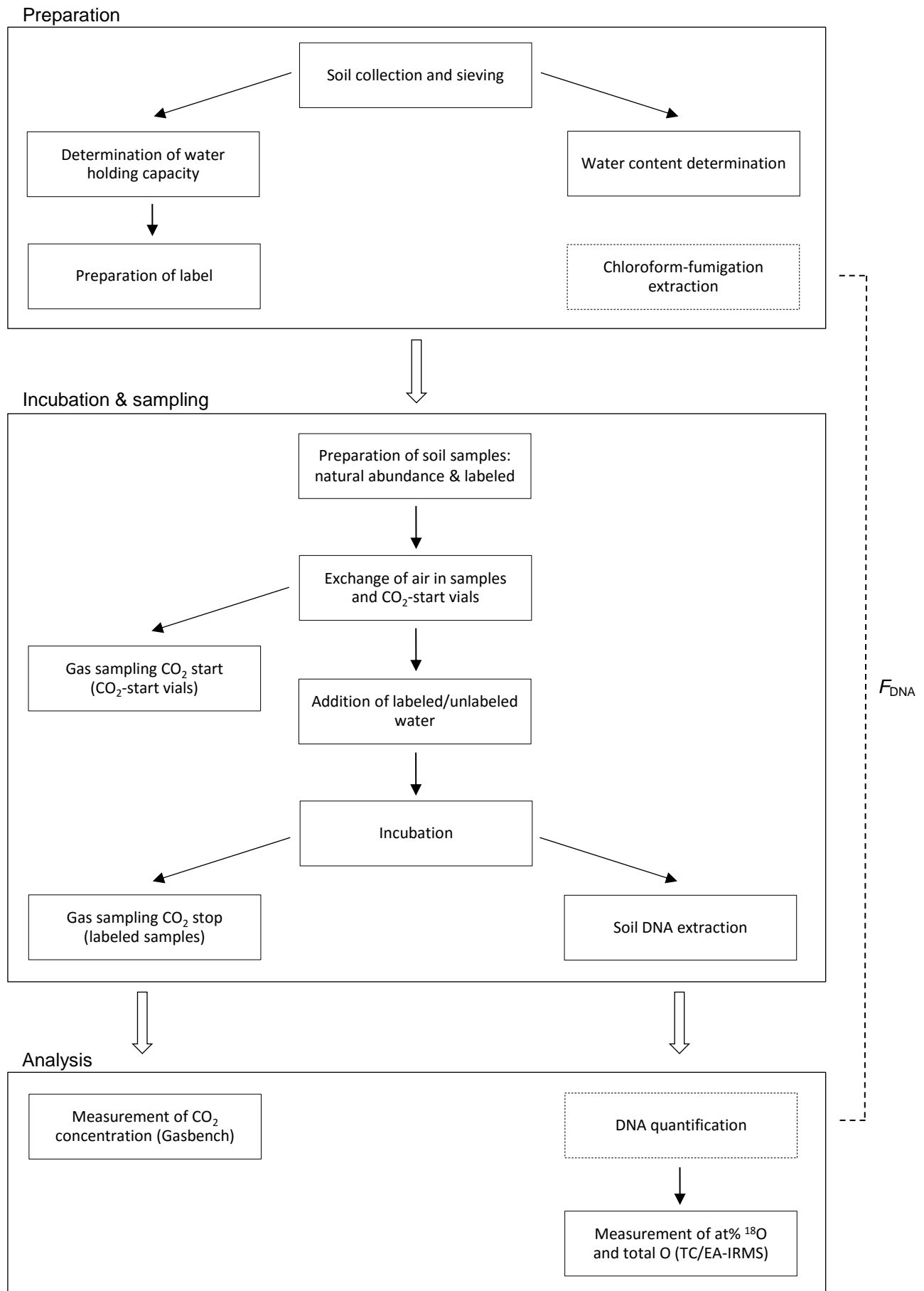
Figures & Tables

Figure 1. Outline of the workflow of the ^{18}O-based method for the determination of soil CUE. Chloroform-fumigation extraction and DNA quantification are required for the determination of a soil-specific conversion factor from DNA to C_{mic} (F_{DNA}).
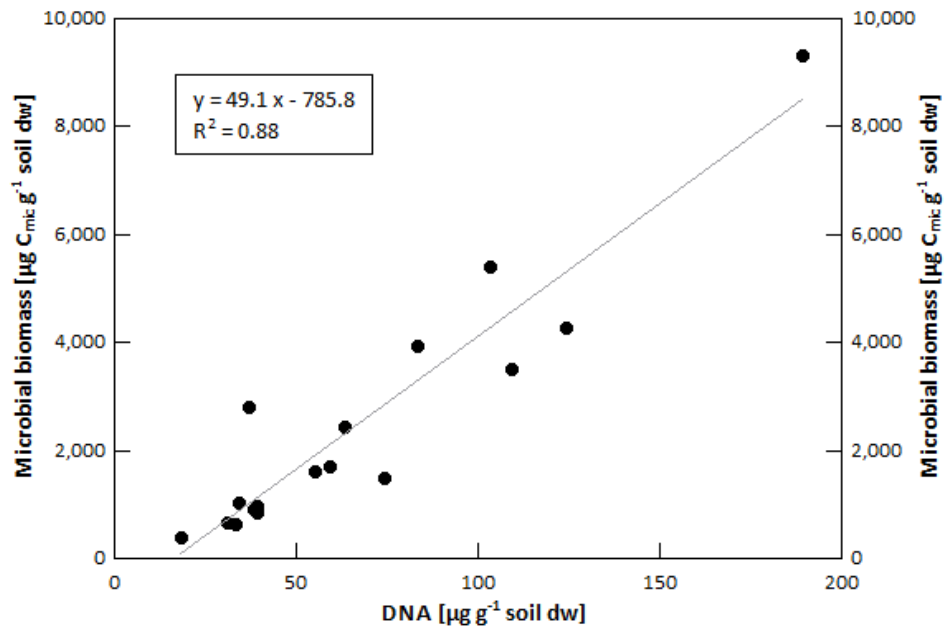
Figure 2. Relationship between extracted *ds*DNA and microbial-biomass carbon (C_{mic}) in an extraction series with high-C_{mic} soils (ten soils > 1500 µg C_{mic} g^{-1} dw), where 500 mg of fresh soil were used for all DNA extractions. The regression is based on 17 soils: four field and arable soils, seven forest soils, and six grassland soils (k = 49.1, r^2 = 0.88, p < 0.001).
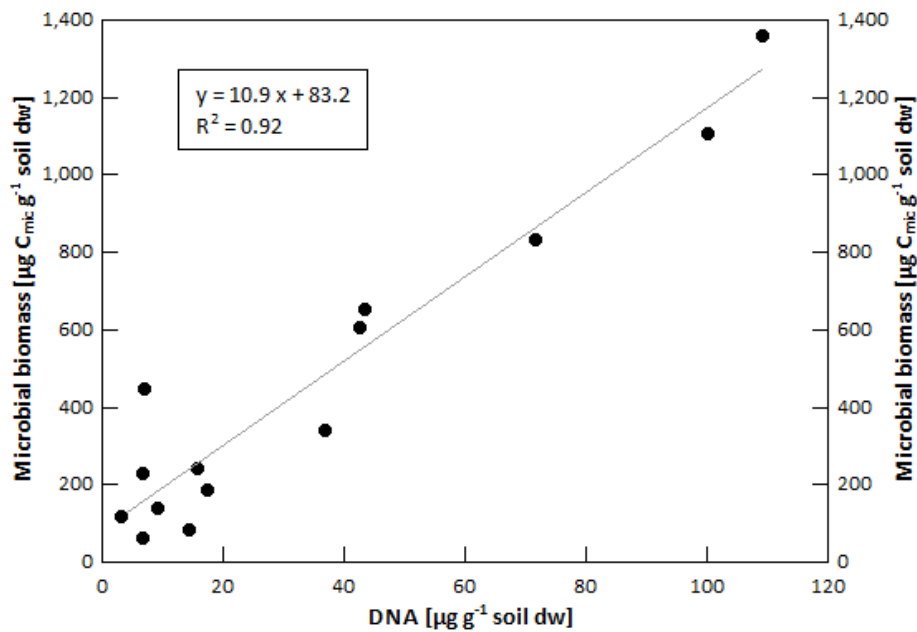

Figure 3. Relationship between extracted *ds*DNA and microbial-biomass carbon (C_{mic}) in an extraction series with low-C_{mic} soils (all soils < 1500 µg C_{mic} g^{-1} dw), where soil weight per DNA extraction was adapted to soil density and expected C_{mic} (and DNA) content: 200 mg of soil for upper soil depths, 500 mg for lower soil depths (n = 14). The regression is based on three soil profiles from an entic podsol, luvic stagnosol, and podsolic stagnosol (each sampled at four depths) and two arable soils (k = 10.9, r^2 = 0.92, p < 0.001). Both *ds*DNA and C_{mic} values are the means of four replicates. Two O horizons of the entic podsol and podsolic stagnosol were identified as outliers (boxplot in R) and excluded from the analysis.
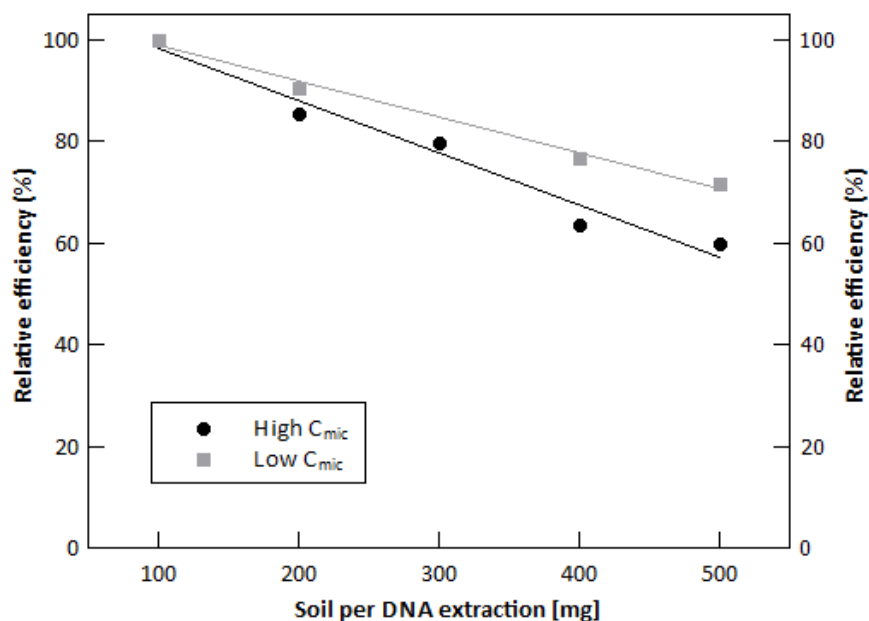
Figure 4. Relative efficiency of soil DNA extraction as a function of the amount of soil that is used for the extraction. Extraction efficiency is assumed to be 100% with 100 mg of soil; the extraction efficiency of other extractions is expressed as percentage of extracted DNA g^{-1} soil dw relative to extracted DNA g^{-1} soil dw from 100 mg soil. Extractions were done with two mineral soils: a luvic stagnosol sampled at a depth of 3-7 cm with a C_{mic} content of 1109 µg g^{-1} dw ("High C_{mic}"; dots and linear regression line in black; n = 5), and an entic podsol sampled at a depth of 10-15 cm with a C_{mic} content of 64 µg g^{-1} dw ("Low C_{mic}"; squares and linear regression line in grey; n = 4). The relative extraction efficiency decreases with increasing amounts soil for both soils, with a more pronounced decrease for the high-C_{mic} soil.
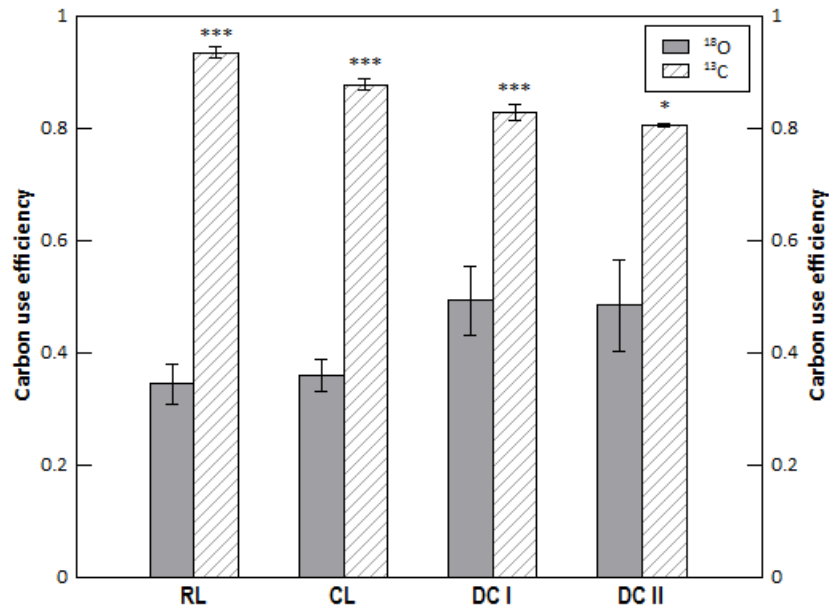
Figure 5. Carbon use efficiency derived from ^{18}O-based and ^{13}C-based method. The two experiments were performed in parallel with four different mineral soils collected in Gumpenstein, Austria: a rendzic leptosol (RL), a cambic leptosol (CL), and two dystric cambisols (DC I and DC II). Each soil was incubated at 15°C in the dark for 24 hours with ^{18}O-labeled water or ^{13}C-labeled glucose solution, respectively. Values are the means of four replicates (error bars: standard deviation). Asterisks indicate significant differences between CUE determined by ^{18}O method and CUE by ^{13}C method according to ANOVA and Tukey HSD (or, where conditions were not met, Kruskal-Wallis Test in conjunction with Mann-Whitney-Wilcoxon Test): $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).
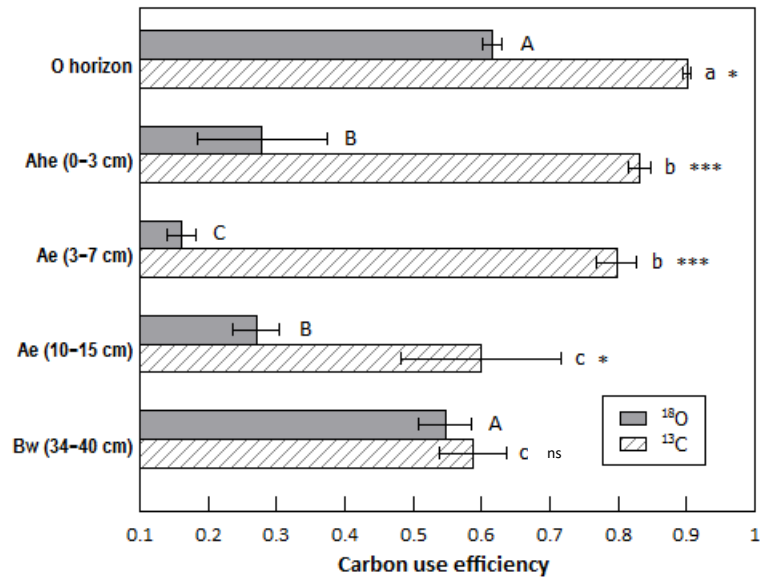
Figure 6. Carbon use efficiency derived from ^{18}O-based and ^{13}C-based method in an entic podsol soil profile from a coniferous forest in Bayreuth, Germany. Samples were taken from the O horizon and four different depths: 0-3 cm (Ahe horizon), 3-7 cm and 10-15 cm (Ae horizon), and 34-40 cm (Bw horizon). Each soil was incubated at 15°C in the dark for 24 hours with ^{18}O-enriched water or ^{13}C-labeled glucose solution, respectively. Values are the means of four replicates (error bars: standard deviation), except for the two lowest horizons with the ^{18}O method (means of three replicates). Letters indicate significant differences between the soil horizons (capital letters: ^{18}O method; minor letters: ^{13}C method) (p < 0.05) according to ANOVA and Tukey HSD (or, where conditions were not met, Kruskal-Wallis Test in conjunction with Mann-Whitney-Wilcoxon Test). Asterisks indicate significant differences between CUE determined by ^{18}O method and CUE by ^{13}C method: p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), not significant (ns).

Table 1. Microbial growth and respiration [ng C h^{-1} g^{-1} soil dw] derived from ^{18}O-based and ^{13}C-based method. Respiration was measured as total respiration for the ^{18}O-based method and as respiration from labeled substrate for the ^{13}C-based method. The two experiments were performed in parallel with four different mineral soils collected in Gumpenstein, Austria: a rendzic leptosol (RL), a cambic leptosol (CL), and two dystric cambisols (DC I and DC II). Each soil was incubated at 15°C in the dark for 24 hours with ^{18}O-enriched water or ^{13}C-labeled glucose solution, respectively. Values represent means of four replicates (in brackets: standard deviation). Asterisks indicate significant differences between growth/respiration obtained by ^{18}O method and growth/respiration obtained by ^{13}C method according to ANOVA and Tukey HSD (or, where conditions were not met, Kruskal-Wallis Test in conjunction with Mann-Whitney-Wilcoxon Test): $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), not significant (ns).

Soil	Growth		Respiration	
	^{18}O method	^{13}C method	^{18}O method	^{13}C method
RL	388 (±29)	581 (±42) ***	750 (±144)	40 (±4) ***
CL	680 (±87)	504 (±17) ***	1124 (±264)	70 (±6) ***
DC I	3732 (±952)	1010 (±105) **	3746 (±348)	209 (±12) *
DC II	330 (±45)	289 (±14) ns	356 (±76)	70 (±4) ***

Table 2. Carbon use efficiency derived from ^{18}O-based and ^{13}C-based method performed with the same soil at two different incubation times (24 hours or 48 hours). A grassland soil from Vienna (Austria) was incubated at 15°C in the dark for 24 or 48 hours with ^{18}O-enriched water or ^{13}C-labeled glucose solution, respectively. Values given are means of three replicates (in brackets: standard deviation). There was no significant difference between the 24-hour and 48-hour incubation with both methods according to ANOVA (ns = not significant).

	CUE	
	24 hours	48 hours
^{18}O method	0.45 (±0.02)	0.42 (±0.02) ns
^{13}C method	0.85 (±0.03)	0.79 (±0.02) ns

Table 3. Microbial growth and respiration [ng C h^{-1} g^{-1} soil dw] derived from ^{18}O-based and ^{13}C-based method in an entic podsol soil profile from a coniferous forest in Bayreuth, Germany. Respiration was measured as total respiration for the ^{18}O-based method and as respiration from labeled substrate for the ^{13}C-based method. Soil samples were taken from the O horizon and four depths: 0-3 cm (Ahe horizon), 3-7 cm and 10-15 cm (Ae horizon), and 34-40 cm (Bw horizon). Samples were incubated at 15°C in the dark for 24 hours with ^{18}O-enriched water or ^{13}C-labeled glucose solution, respectively. Values are the means of four replicates (in brackets: standard deviation), except for depths 10-15 cm and 34-40 cm with the ^{18}O method (means of three replicates). Asterisks indicate significant differences between growth/respiration obtained by ^{18}O method and growth/respiration obtained by ^{13}C method according to ANOVA and Tukey HSD (or, where conditions were not met, Kruskal-Wallis Test in conjunction with Mann-Whitney-Wilcoxon Test): $p < 0.05$ (*), $p < 0.01$ (**) und $p < 0.001$ (***), not significant (ns).

	Growth		**Respiration**	
	^{18}O method	**^{13}C method**	**^{18}O method**	**^{13}C method**
O horizon	7123 (±468)	826 (±63) ***	4433 (±85)	90 (± 2) ***
Ahe (0-3 cm)	317 (±130)	135 (±8) ***	802 (±225)	27 (±2) ***
Ae (3-7 cm)	54 (±4)	71 (±6) **	289 (±61)	18 (±2) *
Ae (10-15 cm)	32 (±5)	24 (±3) **	86 (±3)	16 (±6) *
Bw (34-40 cm)	74 (±5)	28 (±4) ns	61 (±9)	20 (±3) ***

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III.   APPENDIX

1. Supplementary material

Supplementary Table 1. Comparison of total oxygen (O) yield and at% ^{18}O in dried DNA extracts of ^{18}O-labeled soil samples according to measurement on TC/EA-IRMS. Shown are also the ^{18}O excess compared to natural abundance samples, the total soil DNA content according to Nanodrop measurement (Δ) or PicoGreen quantification (†), and the ratio of total oxygen contained in the dried DNA extracts (according to measurement on TC/EA-IRMS) compared to the oxygen in DNA (according to Nanodrop/PicoGreen quantification). Grassland, forest and arable soil were collected in Vienna, Austria. Rendzic leptosol, two dystric cambisols and cambic leptosol were sampled in Gumpenstein, Austria. The entic podsol (organic layer and four mineral horizons) was collected in Bayreuth, Germany. Values are the means of four replicates (except grassland, arable, forest soil and entic podsol Ahe lower and Bw: three replicates). In brackets: standard deviation.

Soil	IRMS		^{18}O excess		Total DNA	O measured: O expected
	Total O [μg]	^{18}O [at%]	^{18}O [at%]	^{18}O [ng]	DNA [μg g^{-1} soil]	
Grassland soil	122.0	0.212	0.008	9.25 (±0.50)	40.2 (±3.9)^{Δ}	25
Arable soil	127.6	0.207	0.003	3.40 (±0.44)	24.0 (±1.2)^{Δ}	23
Forest soil	133.7	0.214	0.009	12.49 (±1.40)	39.6 (±2.0)^{Δ}	27
Rendzic leptosol	122.7	0.209	0.006	7.40 (±0.55)	49.8 (±4.7)^{Δ}	25
Dystric cambisol I	133.9	0.214	0.009	12.17 (±3.84)	105.5 (±7.2)^{Δ}	22
Dystric cambisol II	133.5	0.216	0.012	16.28 (±2.22)	52.7 (±4.1)^{Δ}	22
Cambic leptosol	122.2	0.213	0.009	34.09 (±4.35)	46.8 (±2.9)^{Δ}	23
Entic podsol O	132.6	0.218	0.014	18.68 (±1.23)	127.3 (±31.3)†	38
Entic podsol Ahe	133.0	0.207	0.004	5.29 (±2.18)	42.3 (±4.2)†	56
Entic podsol Ae upper	143.8	0.208	0.004	5.82 (±0.43)	14.2 (±0.9)†	66
Entic podsol Ae lower	135.9	0.205	0.002	2.19 (±0.37)	6.7 (±0.5)†	131
Entic podsol Bw	139.3	0.204	0.001	1.23 (±0.08)	3.1 (±0.4)†	220

Supplementary Table 2. Comparison of different DNA extraction methods: two commercially available extraction kits (FastDNA™ SPIN Kit for Soil, MP Biomedicals, and PowerSoil® DNA Isolation Kit, Mo Bio Laboratories) and a conventional DNA extraction method („CHL") with varying concentrations of sodium dodecyl-sulfate (SDS). 250 mg of fresh soil from a colline grassland site (collected in Gumpenstein, Austria) were used in all extractions. Shown is the amount of DNA extracted per g fresh soil according to three different measurement methods: Nanodrop ND1000 UV-VIS Spectrophotometer, Qubit Fluorometric Quantitation (Thermo Fisher Scientific), and fluorometric quantification by staining with PicoGreen® (Life Technologies). Nanodrop absorption ratios are shown as an indicator of DNA extract purity: low 260/280 values are an indicator of e.g. proteins in the DNA extract, low 260/230 values of aromatic compounds, e.g. humic substances, or compounds used in the extraction such as ethanol. „O measured : O expected" refers to the ratio of total oxygen contained in the dried DNA extracts (according to measurement on TC/EA-IRMS) compared to the oxygen in DNA according to each respective DNA quantification method. Values are the means of two extraction replicates (except PicoGreen quantification for the conventional method, which was only performed with one of the replicates). For the kit extractions, DNA was extracted according to the manufacturer's protocols. For the conventional method, soil was bead-beaten at 6.0 m/s for 30 sec in a Lysing Matrix E Tube (MP Biomedicals) with a mixture of 300 µL each of chloroform-isoamyl alcohol (24:1), 100 mM HEPES buffer (pH 8), and 100 mM NaCl solution with 10%, 5%, 1.25% [wt/vol] SDS and no SDS, respectively. After centrifugation at 14,000 x g for 3 min, the supernatant was transferred to a fresh Eppendorf tube and mixed with an equal volume of chloroform-isoamyl alcohol (24:1). The phases were mixed by manual inversion and samples were centrifuged again at 14,000 x g for 3 min. The supernatant was transferred to a clean Eppendorf tube, mixed with two volumes of ice-cold ethanol (95%), and stored at -20°C for 1 hour to precipitate DNA. DNA was then recovered by centrifugation at 4°C (14,000 x g) and the DNA pellet was washed three times with 70% ethanol. Finally, DNA was eluted in 100 µL PCR-grade water and the extract was cleaned with OneStep™ PCR Inhibitor Removal Kit (Zymo Research).

DNA extraction	Nanodrop			Qubit	PicoGreen	O measured : O expected		
	DNA [µg g^{-1} soil]	260/280	260/230	DNA [µg g^{-1} soil]	DNA [µg g^{-1} soil]	Nanodrop	Qubit	PicoGreen
FastDNA Kit	40.1	1.7	0.2	25.9	33.6	56.1	89.0	68.3
PowerSoil Kit	1.8	1.8	0.7	1.1	1.0	60.6	110.4	159.2
CHL + 10% SDS	41.3	1.6	0.8	23.9	21.0	5.3	15.5	14.7
CHL + 5% SDS	24.3	1.5	0.7	12.4	5.7	6.3	21.0	20.6
CHL + 1% SDS	49.6	1.5	0.7	20.4	21.9	5.5	22.2	16.3
CHL + 0% SDS	6.5	1.5	0.7	2.1	3.1	5.9	35.2	20.9

Supplementary table 3. Overview of microbial growth and respiration [ng C h^{-1} g^{-1} soil dw] and CUE according to ^{18}O-based and ^{13}C-based method for a range of soils. Respiration was measured as total respiration for the ^{18}O-based method and as respiration from labeled substrate for the ^{13}C-based method. Shown is also the specific conversion factor F_{DNA} for each soil, which was used to determine newly formed microbial carbon from newly formed DNA. All soils were incubated at 15°C in the dark for 24 hours with ^{18}O-enriched water or ^{13}C-labeled glucose solution, respectively. Grassland, forest and arable soil were collected in Vienna, Austria. Rendzic leptosol, two dystric cambisols and cambic leptosol were sampled in Gumpenstein, Austria. The entic podsol (organic layer and four mineral horizons) was collected in Bayreuth, Germany. Values are the means of four replicates (except grassland, arable, forest soil and entic podsol Ahe $_{lower}$ and Bw: three replicates). In brackets: standard deviation.

Soil	^{18}O method			^{13}C method			Conversion factor F_{DNA}
	Growth	Respiration	CUE	Growth	Respiration	CUE	
Grassland soil	546 (±29)	664 (±14)	0.45	225 (±38)	38 (±2)	0.85	33.1
Arable soil	49 (±6)	141 (±5)	0.26	139 (±25)	25 (±1)	0.84	9.5
Forest soil	368 (±41)	656 (±50)	0.36	161 (±55)	55 (±2)	0.74	17.9
Rendzic leptosol	388 (±29)	750 (±144)	0.34	581 (±42)	40 (±4)	0.94	34.7
Dystric cambisol I	3732 (±952)	3746 (±348)	0.49	1010 (±105)	209 (±12)	0.83	47.2
Dystric cambisol II	330 (±45)	356 (±76)	0.48	289 (±14)	70 (±4)	0.81	21.8
Cambic leptosol	680 (±87)	1124 (±264)	0.36	504 (±17)	70 (±6)	0.88	31.1
Entic podsol O	7123 (±468)	4433 (±85)	0.62	826 (±63)	90 (±2)	0.90	44.2
Entic podsol Ah	317 (±130)	802 (±225)	0.28	135 (±8)	27 (±2)	0.83	14.3
Entic podsol Ae $_{upper}$	54 (±4)	289 (±61)	0.16	71 (±6)	18 (±2)	0.80	6.1
Entic podsol Ae $_{lower}$	32 (±5)	86 (±3)	0.27	24 (±3)	16 (±2)	0.60	9.7
Entic podsol Bw	74 (±5)	61 (±9)	0.55	28 (±4)	20 (±3)	0.59	39.3
**MEAN**			_0.39 (±0.13)_			_0.80 (±0.10)_	_25.9 (±13.7)_

2. Zusammenfassung

Die mikrobielle Kohlenstoffnutzungseffizienz (carbon use efficiency; CUE) beschreibt wie effizient Mikroorganismen Kohlenstoff für Wachstum, also die Produktion neuer Biomasse, nutzen. Eine hohe CUE bedeutet, dass ein verhältnismäßig großer Anteil des assimilierten Kohlenstoffs in Wachstum investiert wird; eine niedrige CUE folglich, dass ein größerer Teil zu CO_2 veratmet wird. Da jener Anteil des Kohlenstoffs, der in mikrobielle Biomasse eingebaut wird, das Potenzial hat für längere Zeiträume im Boden zu verbleiben, ist die CUE ein ausschlaggebender Faktor für Kohlenstoffspeicherung und ein wichtiger Teil von biogeochemischen Modellen, die sich mit Ausmaß und Auswirkungen des Klimawandels beschäftigen. Aus thermodynamischen Erwägungen ergibt sich, dass die CUE maximal 0,6 betragen kann, dass also maximal 60% des aufgenommenen Kohlenstoffs für Wachstum verfügbar gemacht werden können. Da in der Umwelt allerdings selten optimale Bedingungen für Wachstum herrschen, ist davon auszugehen, dass der globale Durchschnitt der CUE wesentlich niedriger, nämlich bei etwa 0,3, liegt. Das steht in Kontrast zu Messungen der CUE in Böden, die einen Durchschnittswert von 0,55 ergeben. Diese Diskrepanz ist vermutlich darauf zurückzuführen, dass die Standardmethode zur Messung von CUE in Böden – die Inkubation mit einem ^{13}C-markierten Substrat und Nachverfolgung des ^{13}C in mikrobielle Biomasse und CO_2 – mehrere Nachteile hat, die in Summe wohl zu einer Überschätzung der CUE führen. In der vorliegenden Arbeit wird ein neuer Ansatz zur Messung der CUE in Böden beschrieben, der auf der Inkubation mit ^{18}O-markiertem Wasser beruht. Mikrobielles Wachstum wird bei der neuen Methode aus der Inkorporation von ^{18}O in DNA bestimmt. Dieser Ansatz hat gegenüber der Standardmethode mehrere Vorteile: (a) er basiert auf der Verwendung aller bereits natürlich im Boden vorhandenen Substrate, nicht eines einzelnen hinzugefügten Substrats; (b) Wachstum wird nicht aus Biomasseinkorporation eines labilen Substrates, sondern aus der Bildung neuer DNA bestimmt; (c) der Eintrag von Wasser ist ein natürliches Phänomen in terrestrischen Ökosystemen und (d) alle Mikroorganismen nehmen Wasser auf. Wir testeten unsere neue Methode an sieben verschiedenen Böden und einem Bodenprofil mit fünf Horizonten; zum Vergleich führten wir jeweils auch die Standardmethode mit ^{13}C durch. Die mit der neuen Methode bestimmte durchschnittliche CUE lag bei 0,39 (± 0,13), während die Standardmethode eine CUE von 0,80 (± 0,10) ergab. Wir schließen daraus, dass unsere Methode eine sinnvolle Alternative zur Standardmethode ist und CUE-Werte ergibt, die in weitaus besserer Übereinstimmung mit theoretischen Erwägungen stehen. Wir erwarten daher, dass die neue

Methode wichtige Aufschlüsse über die CUE in terrestrischen Ökosystemen im Licht des Klimawandels geben wird.

3. Curriculum vitae

KAROLINE KLAUS

Born 17 March 1982

EDUCATION

Since 10/2012	Master in Environmental Sciences at the University of Vienna
10/2009 – 11/2012	Bachelor in Biology (Ecology) at the University of Vienna
09/2006 – 09/2007	European Master in Human Rights and Democratisation at the European Inter-University Centre for Human Rights (Venice, Italy) and Åbo Akademi (Turku, Finland)
10/2000 – 04/2006	Diploma in Law at the University of Vienna
07/2004 – 07/2005	Erasmus Studies at the University of Oslo (Norway)
09/1992 – 06/2000	Bundesgymnasium Franklinstraße 21 in Vienna
09/1988 – 06/1992	Elementary school in Wolkersdorf im Weinviertel

PROFESSIONAL EXPERIENCE

08/2008 – 08/2015	General Settlement Fund of the Republic of Austria for Victims of National Socialism, Vienna
01/2008 – 07/2008	Federal Ministry for Europe, Integration and Foreign Affairs, Department of Human Rights and Humanitarian Law, Vienna (sponsored internship)
07/2005 – 07/2006	Domestic Abuse Intervention Centre, Vienna

STUDY-RELATED WORK EXPERIENCE

05/2015 – 07/2015	Internship at the EU Environmental Bureau (Umweltdachverband, Vienna)
03/2015 – 06/2015 03/2014 – 07/2014	Tutor at the Division of Terrestrial Ecosystem Research, Department of Microbiology and Ecosystem Science, University of Vienna
10/2012 – 02/2013	Tutor at the Institute of Organic Chemistry, University of Vienna
08/2011 – 09/2011	Internship at the Estación Científica San Francisco (Loja, Ecuador)

LANGUAGES

- English (fluent)
- Norwegian (good)
- French (basic)
- Italian (basic)