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The Effect of Crop Diversification and Season on Microbial Carbon Use Efficiency Across a European Pedoclimatic Gradient

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ABSTRACT

Microbial transformation of soil organic matter plays a critical role in carbon (C) cycling making it essential to understand how land use and management practices influence microbial physiology and its connection to C dynamics. One factor that is likely to impact soil microbial physiology is crop diversification via its influence on belowground diversity (e.g., chemical heterogeneity of C inputs, microbial community composition). However, the effect of crop diversification measures on microbial physiology and potential effects on C cycling in agricultural soils is still unclear. To address this knowledge gap, we sampled topsoil from eight experimental sites covering different crop diversification measures across Europe (i.e., cover crops, ley farming, vegetation stripes). We used the ^{18}O-labelling method to analyse microbial C use efficiency (CUE), growth, respiration and biomass C. Additionally, a second sampling at five selected sites examined whether the growing season influenced the impact of crop diversification. Meta-analysis revealed no overall effect of crop diversification on CUE, microbial activity, biomass or soil organic C (SOC). However, the effects varied with the type of diversification measure: cover crops did not affect carbon processing, vegetation stripes increased microbial activity, and ley farming enhanced CUE. The largest variation in CUE was observed between samplings at the same sites, indicating seasonal dynamics. Temperature, precipitation and photosynthetically active radiation predicted seasonal variation in CUE ($R^2 = 0.36$). While cover crops did not significantly impact C storage in our study, both ley farming and vegetation stripes increased SOC. The overall effect of crop diversification on SOC seems to be decoupled from highly temporally variable CUE in the bulk soil and rather relate to C-inputs.

Summary

- It is unknown whether crop diversification affects microbial C transformation.
- This pan-European study evaluated crop diversification effects on microbial CUE.
- Microbial CUE did not change with crop diversification in general, but varied with season.
- Microbial activity can increase C transformation independent of microbial physiology.

1 | Introduction

To mitigate climate change and simultaneously promote soil fertility, soil organic carbon (SOC) stocks must be preserved and increased. Microbial decomposition and transformation drive SOC dynamics, with microbial metabolic performance being considered a key control (Liang et al. 2017; Soong et al. 2020). In this context, microbial carbon use efficiency (CUE) measures the proportion of total C uptake that microbes invest in growth rather than respiration (Manzoni et al. 2012). A high CUE is expected to reduce CO_2 losses per unit of C that is taken up by microbes and thus support efficient C conversion into microbial biomass. This may positively affect overall SOC storage in the long-term. Microbial debris is a key component of stable soil organic matter because a fraction of microbial-derived compounds and necromass eventually interact with mineral surfaces, forming mineral-associated organic matter which is considered the most stable form of SOC. Thus, microbial C transformation into biomass likely contributes to stabilisation of SOC (Kallenbach et al. 2016; Liang et al. 2020). Due to the critical role of microbial C transformation in C cycling, the influence of land use and management practices on microbial physiology and its connection to C dynamics is becoming increasingly important (e.g., Malik et al. 2018; Poeplau et al. 2019; Schroeder et al. 2024). Knowledge about management effects on CUE is a key component in controlling C dynamics in climate-smart agroecosystems.

Crop diversification measures are a potentially attractive practice for enhancing SOC storage in agricultural soils. Such measures may include the incorporation of an additional plant species into the crop rotation either sequentially or spatially (i.e., intercropping or the growth of cover crops). They may affect SOC in various ways, one of which being altered microbial physiology and/or community composition due to altered composition of carbon input (De Graaff et al. 2010; Domeignoz-Horta et al. 2024). Microbial community composition and the quality of soil organic matter are important drivers of CUE (Sinsabaugh et al. 2016; Soares and Rousk 2019) and both are likely to be influenced by crop diversification. Based on the assumption that a higher aboveground plant diversity produces soil organic matter with greater molecular diversity (Lehmann et al. 2020), there are two contrasting potential mechanisms how and why CUE changes in response to crop diversification: (1) higher molecular diversity creates more microbial niches, thus the community overall can make use of the available C more efficiently, that is, CUE increases; and (2) the higher diversity of inputs results in a less specialised community, which does not necessarily need to feed efficiently on the available substrates, that is, CUE decreases. Thus, crop diversification may alter microbial CUE and C transformation with unknown outcome.

Knowledge of how crop diversification alters microbial CUE in arable soils is scarce (e.g., Bölscher et al. 2016; Liu et al. 2023). The results of Domeignoz-Horta et al. (2024) suggest that plant diversity in a barley-intercropping system may indeed have a positive effect on rhizosphere CUE by increases in stand plant biomass and positive effects on microbial community network connectivity. However, it is unclear to which extent positive effects of plant diversification would extend to the bulk soil. Diversification of grassland species did not result in changes in bulk CUE (Prommer et al. 2020). A positive correlation was reported between tree biodiversity and bulk CUE in a subtropical forest (Duan et al. 2023). In that particular study, α-diversity of tree species (Shannon index) explained 18% of variation in CUE (positive correlation) along a natural diversity gradient (45 plots at 20 m × 20 m). However, given that tree diversity at this natural gradient is also a result of differences in soil properties and microclimate, the isolated effect of aboveground plant diversity on CUE remains uncertain. For example, CUE was positively correlated to soil pH ($R^2 = 0.33$), which varied along a diversity gradient (range 5.8–7.6) and explained more variation in CUE than tree species diversity (Duan et al. 2023). When aboveground plant diversity is increased at arable field-scale, soil properties are either unaltered (e.g., texture) or are subject to changes in other management practices rather than diversification itself (e.g., soil pH, nutrient status). Changes in CUE with crop diversification measures may be more directly linked to changes in aboveground plant diversity. Here, we investigate changes in bulk soil CUE as this is the spatial scale which needs to be targeted for increasing SOC stocks. Crop diversification is a very broad concept and has a spatial (inclusion of an additional crop species during the same time, e.g., intercropping) and temporal component (inclusion of an additional crop species into the crop rotation, e.g., cover crops) (Messéan et al. 2021). In the latter case, also the physiological response of the microbial community to crop diversification could have a distinct interannual dynamic. For example, CUE may change in response to cover crops, but only during the time when cover crops are actually growing. In addition, seasonal patterns of CUE may arise, with higher CUE during cold seasons (Schnecker et al. 2023). It is unclear whether the potential effects of crop diversification measures on CUE exceed seasonal variation of CUE and whether changes in bulk CUE may be linked to SOC dynamics. Therefore, this study investigated whether crop diversification measures have an impact on bulk CUE, how these effects vary by season, and whether changes in microbial physiology due to crop diversification measures can be linked to changes in SOC.

Europe has a pedoclimatic gradient with diverse agricultural systems and crop diversification measures differ accordingly. The most common measure of crop diversification in Central and Northern Europe are cover crops introduced into crop rotations of annual crops, mostly cereals (Eurostat 2022). Cover crops as such are less common in Southern Europe (Fendrich et al. 2023). This may be partly related to the scarcity of water (Blanchy et al. 2023), but also to perennial crops such as olive, fruit or vine orchards, which cover large agricultural areas and are a substantial part of Mediterranean agroecosystems. In

Spain, for example, permanent crops account for about 20% of the agricultural area (Eurostat 2022). In perennial crop systems, crop diversification can be implemented as vegetation cover stripes (e.g., grass or natural vegetation) as compared to bare fallow. Further measures of crop diversification aiming to increase C accrual include the introduction of temporal grassland into rotations of annual crops, known as ley farming. In this context, France's National Research Institute for Agriculture, Food and Environment (INRAE) recommends ley farming and cover crop systems as effective measures to support SOC accrual (Launay et al. 2021). Additionally, the introduction of vegetation stripes in permanent crops is considered moderately beneficial for SOC accumulation (Pellerin et al. 2019). Given their potential positive effect on SOC, these measures are suitable for investigating the link between crop diversification, microbial physiology and C storage.

Our objective was to investigate whether crop diversification measures alter microbial CUE across a pan-European pedoclimatic gradient, thereby potentially influencing C storage. We aimed to cover the general effect of crop diversification on microbial CUE in arable soils and therefore included long-term field sites representative for different crop diversification measures across Europe, mimicking the complexity of real-farm scenarios. In addition, we covered the temporal component of crop diversification effects on CUE by analysing samples taken in two different seasons. To best of our knowledge, this study is the first to investigate the combined impact of crop diversification measures and seasonal variability on microbial CUE in arable soils at a pan-European scale.

2 | Materials and Methods

2.1 | Site Selection and Sampling

In total, eight field experiments along a pan-European pedoclimatic gradient were chosen to represent crop diversification across Europe (Figure 1). Sites had to include at least one diversified treatment representative of crop diversification practices

FIGURE 1 | The selected experimental sites along the pan-European pedoclimatic gradient cover different measures, that is, cover crops (violet), ley farming (green), and vegetation stripes (yellow) representative for crop diversification across Europe.

TABLE 1 | Selected experimental sites across the pan-European pedoclimatic gradient.

Country	Experiment	EPSG 4326		MAT (°C)	MAP (mm)	Growing season (days)	Köppen Geiger
		Latitude	Longitude				
Sweden	Mellby R0-8403	56.496	13.04	7.7	925	242	19
Lithuania	Dotnuva LAMMC cover crops	55.413	23.864	7.2	615	218	19
The Netherlands	Wageningen Clever Cover Cropping	51.995	5.659	10.2	888	365	10
Czech Republic	Prage Ruzyně	50.085	14.301	8.9	578	269	19
Austria	St. Florian	48.212	14.383	9.8	856	280	19
France	Lusignan	46.413	0.12	11.9	878	365	10
Slovenia	Koper	45.5[a]	13.7[a]	14.3	1529	365	9
Spain	Seville Benacazón	37.343	−6.229	18.5	600	256	12

Note: Climate data source: CHELSA Bioclim (Karger et al. 2017; Karger et al. 2018).
[a]No permission to share exact coordinates; rounded to one decimal.

typical in the respective region, as well as a non-diversified control. Mean annual temperature (MAT) and precipitation (MAP) ranged from 8.9°C to 18.5°C and 578 to 1529 mm, respectively (Table 1). Vegetation length varied between 365 (e.g., the Netherlands) and 218 days per year (Lithuania) (Table 1). Using a principal component analysis including climate and soil parameters, the sites could be clearly separated, which confirmed the successful establishment of a pedoclimatic gradient by our site selection (Figure S1). Crop diversification measures included introduction of (i) cover crops or (ii) temporal grassland in annual crop rotations (the latter hereafter referred to as ley farming), and (iii) introduction of vegetation stripes between perennial crops (Figure 1). Table 2 provides an overview on the experimental layout of each field experiment as included in this study. For further details on experimental sites, see the available literature: Sweden (e.g., Aronsson and Torstensson 1998; Poeplau et al. 2015), the Netherlands (e.g., Elhakeem et al. 2023), Czech Republic (e.g., Hakl et al. 2021), and France (e.g., Hu and Chabbi 2022). Detailed information on the management in the years of sampling at each site is given in Table S1.

To cover the temporal dimension of crop diversification, samples were taken twice during the vegetation period (Table S1). More specifically, for cover crop sites samples were taken (i) in early spring and prior to the establishment of the main crops (i.e., April) and (ii) in late autumn when the plant biomass of the cover crop was expected to be highest (i.e., November or December). A third sampling was conducted in the Netherlands (i.e., August), which served as the core site within the project. This sampling was a pre-test, but since the data was valuable, it was included in this study. The repeated, temporal sampling took place at five out of eight locations. This restriction was due to time and access limitation, but sampling in different seasons included sites representative for different environmental conditions and crop diversification measures (Table S1). Topsoil was sampled from 0 to 20 cm. At each plot, 5–20 cores (depending on surface area and soil core diameter) were taken in a w-shaped sampling and combined into one representative composite sample. After manual removal of large organic material and stones, the composite sample was homogenised using a trowel. The samples were shipped to the Thünen Institute in Braunschweig (Germany) on cool pads and stored at −20°C thereafter. Prior further analysis, soil samples were thawed and sieved to 2 mm.

2.2 | General Soil Parameters

Bulk soil total organic C and total N content were determined on dried (60°C) and ball-milled aliquots by dry combustion (TOC-VCPH/CPN/TNM-1 analyser). Additionally, samples with soil pH > 6.5 were analysed for carbonates via stepwise combustion at 450°C for 12 h (TOC-VCPH/CPN/TNM-1 analyser). Water holding capacity was quantified by soaking 10 g soil (40°C oven-dried) placed on a cotton wool-padded funnel with water. The water content quantified when water runoff stopped was assumed to represent 100 %WHC. Soil pH was measured in a 1:5 w/v ratio of soil to H_2O (1 h shaking horizontally, 200 rpm) on 40°C oven-dried samples. Texture and bulk density data was provided by the respective site managers (Table 3). Total organic carbon, total N and water holding capacity were determined on the same topsoil samples obtained for CUE measurements. Table 3, presents a mean ± standard deviation of all samplings, treatments and blocks. Single plot measurements can be retrieved from the raw data provided in the accompanying Zenodo repository.

2.3 | Determination of ^{18}O-CUE

We used the ^{18}O-CUE method as described by Spohn et al. (2016), with the same modifications described in Poeplau et al. (2019) and Schroeder et al. (2021). In the ^{18}O-labelling method, microbial growth is determined based on the incorporation of ^{18}O-labelled water into newly formed DNA (Schwartz 2007). In contrast to conventional ^{13}C-labelling methods where the C substrate added could affect microbial activity and metabolic efficiency, no additional C is added when the ^{18}O-water is added to the soil sample. Therefore, it is a valuable method to determine

TABLE 2 | Experimental layout of experimental sites, with the treatments considered for this study.

Site ID	Land use	Established in	Baseline and treatments	$n_{replicates}$	n_{total}	ctrl	div
Cover crops							
Sweden	Cropland	1983	Spring grain dominated crop rotation C: w/o catch crop; D: with ryegrass	3	6	C	D
Lithuania	Cropland	2022	0: control bare soil; 2: mustard	4 (2); 1 (0)	5	0	2
The Netherlands	Cropland	2016	Wheat-maize (2022)-potato-barley-pea-barley crop rotation Fallow: crop rotation w/o cover crop; crop rotation with cover crops as follows V: vetch; R: radish; O: oats; VO: vetch, oats; VR: vetch, radish; OR: oats, radish; VOR: vetch, oats, radish	5	40	Fallow	VOR
Czech Republic	Cropland	2021	Spring barley followed by different cover crops CT: control SA: cover crop = *sinapis alba* SA + F: cover crop = *sinapis alba* + fagopyrum	4	12	CT	SA + F
Austria	Cropland	2020	Barley-maize crop rotation fallow: crop rotation w/o cover crop; crop rotation with cover crops as follows white lupine; aqua pro: phacelia, linseed, sunflower, bristle oat, niger, sorghum, safflower	3	9	Fallow	Aqua pro
Ley farming							
France	Cropland; ley; grassland	2005	T1: maize, wheat, barley since 2005; T2: 3-year grassland followed by 3-year crops sequence; T5: permanent grassland since 2005	4	12	T1	T2
Vegetation stripes							
Slovenia	Perennial crop	1997	Vineyard with different interrow management BC: bare cover with tillage to control weeds; PG: permanent natural vegetation cover	4	8	BC	PG
Spain	Perennial crop	2009	Olive orchard with different interrow management CT: bare cover with surface tillage to control weeds MC: mixed natural vegetation cover	2 (CT); 4 (MC)	6	CT	MC

Note: Sites are grouped according to diversification measures. To assess the effect of crop diversification on microbial carbon use efficiency along the European gradient, reference (*ctrl*) and diversified (*div*) treatment were selected as indicated.

TABLE 3 | General soil parameters: Soil texture, bulk density (BD), water holding capacity (WHC), soil organic carbon content (SOC) and soil C:N ratio.

Site ID	Clay (%)	Silt (%)	Sand (%)	BD ($g\,cm^{-3}$)	pH	WHC (% w/w)	SOC ($g\,kg^{-1}$)	CN
Sweden	6	43	46	1.24	6.0 ± 0.1	47 ± 3	31.6 ± 4.7	23 ± 1
Lithuania	11	39	50	1.31	7.5 ± 0.1	60 ± 3	25.9 ± 5.9	11 ± 1
The Netherlands	2	12	83	1.49	6.1 ± 0.2	34 ± 4	13.2 ± 5.2	15 ± 3
Czech Republic	23	64	13	1.35	8.2 ± 0.1	58 ± 5	19.6 ± 1.2	11 ± 1
Austria	19	76	5	NA	7.0 ± 0.4	51 ± 4	11.8 ± 1.0	15 ± 3
France	18	66	17	1.47	6.4 ± 0.4	51 ± 4	11.5 ± 1.5	12 ± 3
Slovenia	22	45	33	1.38	8.4 ± 0.1	51 ± 7	14.3 ± 5.6	11 ± 3
Spain	17	37	46	1.17	8.5 ± 0.1	39 ± 4	10.9 ± 2.7	15 ± 4

Note: Values are given as mean ± standard deviation.

the metabolic efficiency during decomposition of soil organic matter (Geyer et al. 2019). This was particularly useful in this study, where crop diversification was hypothesised to affect CUE by greater diversity of chemical compounds and thus by altering organic matter.

Briefly, two aliquots (approx. 350 mg dry weight) of pre-incubated soil samples (1 week at 15°C, water content adjusted to 45% of water holding capacity (WHC)) were weighed into Eppendorf vials, placed into 20 mL glass vials (WICOM Germany GmbH) and crimp-sealed. The amount of ^{18}O-water (80 at% ^{18}O, diluted from 97 at% ^{18}O) needed to adjust a label of 20 at% and a water content of approx. 60%WHC in the final soil solution was added to one of the aliquots through the septum using a syringe (Hamilton). The headspace atmosphere of this vial was then replaced immediately (within 1 min after water addition) with a standard gas of known CO_2 concentration (350 ppm) by evacuating the vial (drop in pressure to 2 mbar) and flushing with standard gas adjusting a pressure of 1300 mbar. To assess the initial CO_2 concentration, standard gas blanks ($n = 3$) were included. Bi-distilled water was added to the second, non-labelled aliquot at the same amounts as to the labelled aliquot. Samples and blanks were incubated for exactly 24 h at 15°C in the dark before taking a gas sample (20 mL) from the labelled vial using a 25 mL gas-tight syringe (SGE Syringe, Trajan Scientific and Medical). Gas samples were analysed for CO_2 concentration using a gas chromatograph equipped with an GC PAL autosampler (CTC Analytics) and a helium ionisation detector for CO_2 detection at an injection volume of 1 mL and an oven temperature of 220°C (Agilent 7890A GC, Agilent Technologies). Subsequently, vials were de-crimped, Eppendorf vials closed and soil was directly frozen in liquid nitrogen and stored at −80°C until the extraction of the DNA.

The DNA was extracted from the whole sample (350 mg) using the FastDNA SPIN Kit for Soil (MP Biomedicals) following the standard protocol, with an extension of the centrifugation to 15 min in step five (15,000 rpm, Sigma 4-16KS). The DNA concentration in the extracts was quantified with the QuantiT PicoGreen dsDNA Kit (Invitrogen). Subsamples of 60 µL of the DNA eluate were transferred to silver capsules (IVA Analysetechnik) and oven-dried at 60°C for 12 h. Isotopic analysis of ^{18}O in the dried DNA were conducted on labelled and non-labelled samples using a high-temperature conversion/elemental analyser (TC/EA) (Thermo Fisher Scientific) coupled with a Delta V Plus isotope ratio mass spectrometer via a ConFloIV interface (Thermo Fisher Scientific).

To be able to convert the amount of DNA produced into C directed to growth, the microbial biomass C to total DNA extracted ratio is needed, that is, *f*DNA ratio. Therefore, microbial biomass C was determined for each sample after the pre-incubation by chloroform fumigation extraction (Vance et al. 1987). Fumigation was conducted with excess chloroform ($CHCl_3$) at room temperature for 24 h in the dark. Non-fumigated and fumigated aliquots (5 g) were extracted with 0.5 M K_2SO_4 solution in a 1:4 w/v soil-to-extractant ratio (horizontal shaker, 30 min at 200 rpm), filtered (hw3, Sartorius Stedim Biotech) and stored frozen at −20°C until further analysis. Total organic C in the extracts was determined as non-purgeable organic carbon (NPOC) analysed in a 1:4 v/v extract dilution after removal of total inorganic C by adding 15% HCl in order to adjust to pH 2–3 and outgassing emerging CO_2 for 5 min with artificial air (Dimatoc 2000, DIMATEC Analysetechnik). Microbial biomass C was calculated using an extraction factor $k_{EC} = 0.45$ (Joergensen 1996).

Microbial respiration ($C_{Respiration}$) was calculated from the increase in CO_2 concentration within 24 h incubation using the ideal gas equation according to Equation (1):

$$C_{\text{Respiration}}\left(\text{ng C g}^{-1}\text{soil h}^{-1}\right) = \frac{p\times V}{R\times T}\times M\times\Delta\text{CO}_2 \times\frac{1}{\text{g soil}\times 24\text{ h}}\times 1000 \quad (1)$$

where p is the pressure (kPa) in the vial (130 kPa), V is the volume (L) of the vial headspace, R is the universal gas constant (8.314 L kPa K^{-1} mol^{-1}), T is the temperature (K) at which the standard gas is injected into the vial (293 K), M is the molecular mass of carbon (12.01 g mol^{-1}), and ΔCO_2 is the increase in CO_2 concentration (ppm) during the incubation time of 24 h (h).

Microbial growth (C_{Growth}) was calculated based on the incorporation of ^{18}O from the labelled soil solution into the microbial DNA based on the enrichment, the average proportion of oxygen

in DNA and a sample specific conversion factor from DNA (μg DNA g^{-1} soil) to C_{mic} (μg C g^{-1} soil), that is, *f*DNA according to Equation (2):

$$C_{\text{Growth}}\left(\text{ng C g}^{-1}\text{ soil h}^{-1}\right) = \text{DNA O} \times \frac{\text{DNA }^{18}\text{O}}{\text{enrichment}} \times \frac{100}{31.21} \times f\text{DNA} \times \frac{1}{\text{g soil} \times 24\text{ h}} \times 1000 \quad (2)$$

where DNA O (μg) is the total amount of O in the DNA eluate derived from the isotopic analysis, DNA ^{18}O (at% excess) is the difference in at % ^{18}O between the labelled and the unlabelled natural abundance control samples, and the *enrichment* of the final soil solution is adjusted to 20 at% ^{18}O. The average % w/w of O in DNA is 31.21 ($C_{39}H_{44}O_{24}N_{14}P_4$).

The microbial carbon use efficiency (CUE) is defined as the share of C directed to microbial growth (i.e., C_{Growth}) to total C metabolised, that is, the sum of *C* directed to growth and respiration (C_{Growth}) (Manzoni et al. 2012):

$$\text{CUE} = \frac{C_{\text{Growth}}}{C_{\text{Growth}} + C_{\text{Respiration}}} \quad (3)$$

2.4 | Data Analysis

Statistical analyses and data visualisation were conducted in R v4.4.0 (2024-04-24 ucrt) (R Core Team 2024) using RStudio v2024.04.0 (Posit team 2024). R packages used are listed in the supplemental material. Unless otherwise stated, values are given as mean ± standard deviation. Data and R code used for this study are freely available at (DOI: 10.5281/zenodo.13271731).

We performed a weighted meta-analysis on the effects of crop diversification on microbial CUE, associated parameters, and SOC, to generalise the effect of crop diversification on microbial C transformation across experimental sites. The meta-analysis approach takes into account the heterogeneity of the experimental sites (e.g., experimental layout, management, soil characteristics, pedoclimatic zones) by treating individual experimental sites as individual studies, representing single observations on the magnitude of the treatment effect, that is, effect size.

The meta-analysis was conducted as three-level random effect meta-analysis using the log response ratio ln(RR) as effect size and weighing by the inverse variance. Some of the studied sites included more than one diversified treatment (e.g., the Netherlands). To warrant the independence of effect sizes, only one effect size per study should be extracted (Gurevitch and Hedges 1999). Therefore, we selected the control (*ctrl*) and the most diverse (*div*) treatment for the meta-analysis (Table 2). However, since seasonality accounted for higher variation in CUE data than treatment or site, we included all sampling times separately in the meta-analysis. To acknowledge the dependence of effect sizes derived from the same experimental site, *site* was included as a cluster in the random-effect model. Inclusion of a third sampling in the Netherlands, compared to two samplings at other sites, did not introduce bias to the statistical analysis, as the model accounted for data dependency.

We extracted the means of response variables, the number of replicates and corresponding standard deviations for the diversified and control treatments. The used effect size (log response ratio) allows to summarise values with large variation across studies, thus allowing the comparison of the effect sizes of different response variables (Fohrafellner et al. 2023). For each response variable, the response ratio (RR) was calculated as:

$$\text{RR} = \frac{\underline{X}_{\text{div}}}{\underline{X}_{\text{ctrl}}} \quad (4)$$

where $\underline{X}_{\text{div}}$ and $\underline{X}_{\text{ctrl}}$ represent the response variable means of the diversified and control treatment, respectively. The statistical analyses were performed on the natural logarithm of RR due to its more normal distribution in small samples compared to that of RR (Hedges et al. 1999; Fohrafellner et al. 2023).

In the random-effect meta-analysis, between-study variance is acknowledged by including an estimate of the variance of the distribution of true effect sizes τ^2 as error term in the inverse variance calculation, which is then used as a weighing factor. The weighted mean of ln(RR) over all studies was calculated as follows (Fohrafellner et al. 2024):

$$\ln(\text{RR}) = \frac{\sum_{i=1}^{n} w_i \times \ln(\text{RR})_i}{\sum_{i=1}^{n} w_i} \quad (5)$$

where $\ln(\text{RR})_i$ is the log response ratio for study *i*, *n* is the number of studies and w_i is the weight for study *i*, defined as the inverse of the sum of variance of study *i* (V_i) and the heterogeneity τ^2 (i.e., between-study variance).

τ^2 was estimated using the restricted estimated maximum likelihood method. Kenward-Roger adjustment was used to generate the 95% confidence intervals (CIs) around the pooled effect sizes. Crop diversification effects were considered significant, if the 95% CI did not overlap with zero and the random-effect model *p*-value was <0.05. The Lithuanian site was excluded from the meta-analysis, due to missing replication of *ctrl*.

High heterogeneity of effect sizes between studies could result in over- or underestimation of the true effect size, for example when one heavier weight study points into a completely different direction than other studies. But also grouping factors, such as diversification measure, could induce between-study heterogeneity. The heterogeneity of effect sizes was tested using Cochrane's *Q* test, with *p*-value <0.05 indicating that the effect sizes vary significantly between studies. The I^2 statistic is defined as the percentage of variability in the effect sizes that is not caused by sampling error, with 25%, 50%, and 75% indicating low, moderate and substantial between-study heterogeneity in effect sizes, respectively (Harrer et al. 2022). We conducted a subgroup analysis with diversification measure as categorical moderator. To test whether response ratios differed between subgroups, that is, diversification measures, we used Cochrane's *Q* test, where $p<0.05$ indicates that diversification measure explains a significant amount of variability in the effect sizes ln(RR). Given the small number of studies for ley farming and vegetation stripes, we fixed between-study (i.e., observations) heterogeneity to a common estimate between subgroups to

avoid imprecise estimates of τ^2 (Harrer et al. 2022). Results were back-transformed and reported as RR.

To assess the effects of sampling time and treatment (independent variables) on microbial CUE (response variable) at each individual site, we employed two-way analysis of variance (ANOVA), including all treatments (Table 2). We used the Shapiro–Wilk test ($p < 0.05$) and visual inspection of the Q–Q plots to test for the normality assumption of residuals, which was fulfilled. According to the Levene test, all data used for ANOVA analysis exhibited homogeneity of variances. To further investigate treatment effects on CUE, C_{mic}, C_{Growth}, $C_{Respiration}$, and SOC at the ley farming site in France—the only site where crop diversification led to a significant change in CUE according to the meta-analysis—a linear mixed-effects model with sampling and treatment as fixed factors was fitted without interaction of these two (no significant difference between models with and without interaction and lower AIC without interaction). *Block* was introduced as a random factor, allowing for a random intercept.

Differences in CUE between samplings could follow seasonal patterns. However, assigning seasons to sampling dates along the pedoclimatic gradient is not trivial: While one can clearly indicate a winter sampling during December in Sweden, weather conditions differ considerably when sampling in December in Spain. To investigate the potential influence of season on CUE, we therefore extracted numerical seasonal predictors. A period of 3 months was chosen to obtain a picture of the environmental conditions that may have shaped the microbial community in the period prior to sampling. We extracted daily weather data for the respective site coordinates from the National Aeronautics and Space Administration (NASA) Langley Research Center (LaRC) Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program, that is, NASA POWER project, for a 3-month period prior to each sampling event: mean daily air temperature at 2 m (T2M), the bias corrected average of total precipitation at the Earth's surface (PRECTOTCORR), and the total photosynthetically active radiation incident at the Earth's surface (ALLSKY_SFC_PAR_TOT).

Figure 2 illustrates the predictor extraction based on 3-month weather data. Mean daily temperature was fitted over time and the slope of the regression extracted as a predictor (slope temperature) to represent the shift from warm to cold season and vice versa. The flatter the curve, the more likely the sampling was carried out during a time of steady temperatures, that is, summer or winter. However, slope temperature alone does not distinguish cold and warm seasons. Therefore, the distance of the mean 3-month temperature to the mean annual temperature was retrieved as additional predictor (Δ temperature), where Δ temperature > 0 and Δ temperature < 0 indicate warm and cold season, respectively. The cumulative precipitation was normalised to mean annual precipitation to represent the relative amount of yearly precipitation having occurred in the time before sampling (Σ precipitation). It has to be noted that the indicator for precipitation does not consider fluctuations in rainfall in the period before sampling. The sample-specific water content referred to dry mass at time of sampling was included as additional information on water availability in the driver analysis. Last, the cumulative photosynthetically active radiation incident over the 3-month period was summed (Σ PAR). We tested the autocorrelation between the potential predictors. The extracted weather predictors confirmed that the two samplings at each of the five sites were indeed conducted at different times of the year, that is, warm and cold season (Table S2).

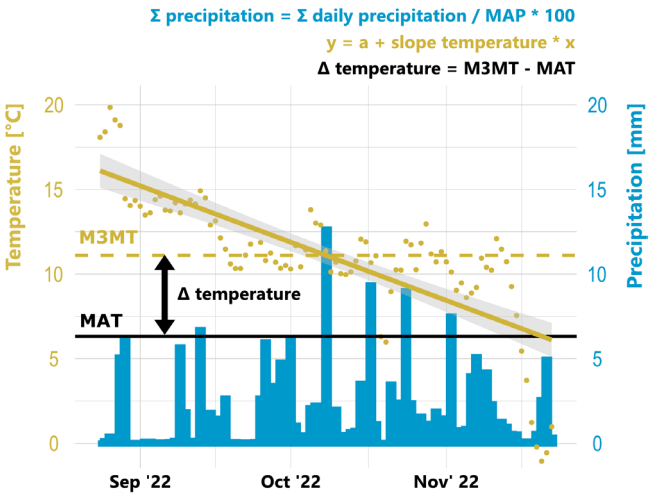

FIGURE 2 | Example of the extraction of seasonal predictors from 3-month weather data prior sampling. The fitted slope over 3-month daily temperatures was extracted (slope temperature [°C d^{-1}]) to represent the direction and extent of temperature change during the time before sampling. The difference between mean 3-month temperature (M3MT) and mean annual temperature (MAT), that is, Δ temperature (°C), identifies cold and warm seasons. The precipitation predictor Σ precipitation (%MAP) is the sum of daily precipitation during 3-month period divided by mean annual precipitation (MAP).

To assess potential seasonal drivers of CUE, we employed a linear mixed-effects model with slope temperature, Δ temperature, Σ precipitation, water content, and Σ PAR as fixed effects, and site as random effect (random intercept). Scales differed between the parameters, for example slope temperature ranged between −4.2°C and 2.4°C per day, whereas Σ precipitation ranged between 8 and 42 %MAP. Therefore, CUE and predictor variables were centred by its mean and normalised by its standard deviation, that is, z-transformed, prior to driver analysis, allowing to assess how much of the variation in the predictor variable explained a certain variation in CUE. A visual inspection of residual plots was used to check for deviations from homoscedasticity or normality. Significance of the fixed effects was assessed at a significance level of $\alpha = 0.05$. The p-values were Tukey corrected.

3 | Results

3.1 | Crop Diversification Effect Across the EU Gradient

Our results revealed that crop diversification had no overall effect on microbial CUE, growth, respiration, biomass C or SOC (Figure 3). However, the heterogeneity of observed effect

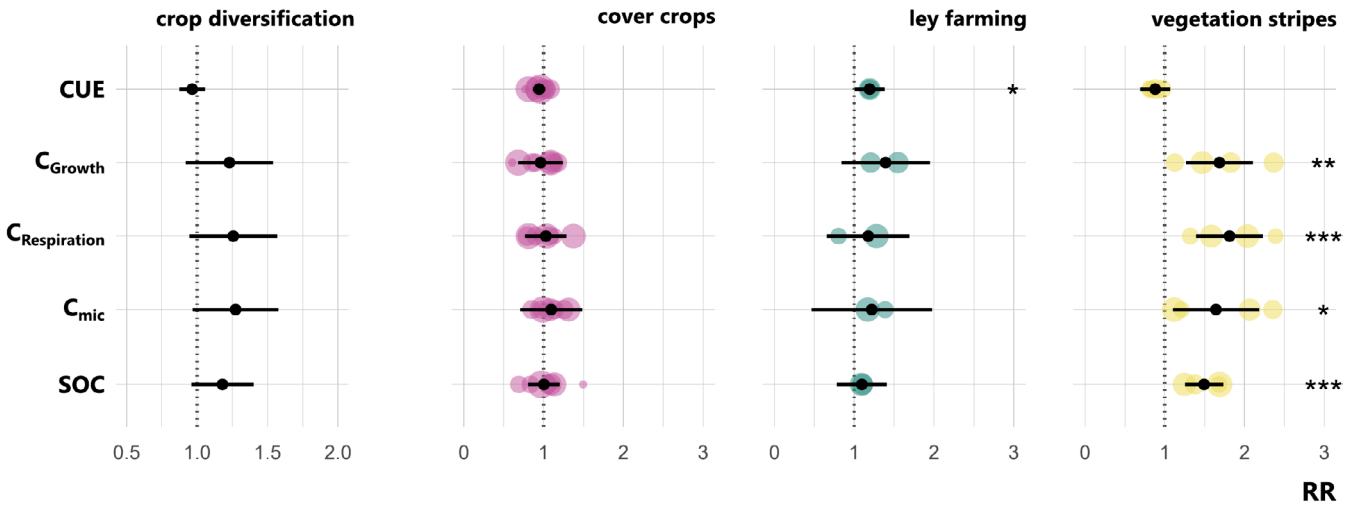

FIGURE 3 | Overall and measure specific effect sizes of crop diversification on microbial carbon use efficiency (CUE), growth (C_{Growth}), respiration ($C_{Respiration}$), biomass C (C_{mic}), and soil organic C (SOC). Effects of crop diversification are given as response ratio (RR), that is, relative change diversified versus control treatment. Coloured circles mark single observations, where the size represents its weighted contribution to the estimated overall effect. Asterisks indicate significant diversification effects (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

sizes was generally high (except for CUE) (Table S3), which was partly explained by differences between diversification measures. CUE effect sizes showed low to moderate heterogeneity across observations ($I^2 = 34\%$, $p_Q = 0.114$), with observed RR ranging from 0.83 to 1.21. However, ley farming was the only measure ($p_{subgroup} = 0.036$) which increased CUE (RR 95% CI: 1.00–1.47, $n = 2$, $n_{sites} = 1$). We observed heterogeneous effects of crop diversification on C_{Growth} ($I^2 = 89\%$, $p_Q < 0.001$) and $C_{Respiration}$ ($I^2 = 90\%$, $p_Q < 0.001$). This was explained by vegetation stripes significantly increasing microbial activity, that is, C_{Growth} (RR 95% CI: 1.48–3.43, $n = 4$, $n_{sites} = 2$) and $C_{Respiration}$ (RR 95% CI: 1.48–3.43, $n = 4$, $n_{sites} = 2$), whereas the other measures showed no effect (C_{Growth}: $p_{subgroup} = 0.015$; $C_{Respiration}$: $p_{subgroup} = 0.008$). Likewise, the effect of crop diversification on microbial biomass C was heterogeneous across our observations ($I^2 = 88\%$, $p_Q < 0.001$), without significant differences between measures ($p_{subgroup} = 0.276$). Regardless, vegetation stripes increased C_{mic} (RR 95% CI: 1.10–3.27, $n = 4$, $n_{sites} = 2$). Crop diversification tended to generally increase SOC ($RR_{overall}$ 95% CI: 0.96–1.49, $n = 14$, $n_{sites} = 7$, Lithuania excluded). However, this overall effect was influenced by the positive effect of vegetation stripes on SOC (RR 95% CI: 1.28–2.09, $n = 4$, $n_{sites} = 2$), which contributed to 33% to the overall effect size. Again, cover crops and ley farming did not alter SOC ($p_{subgroup} = 0.008$), according to the meta-analysis. It has to be noted that effect sizes tended to be higher in Slovenia than Spain, with significant differences between Slovenia and Spain for C_{Growth} and C_{mic}. Thus, the strong positive effects of crop diversification observed in the subgroup of vegetation stripes were likely driven by the Slovenian site. Nevertheless, both sites showed positive effects.

According to the linear-mixed effect model including all treatments at Lusignan (i.e., cropland, ley farming, permanent grassland), CUE ($p = 0.0173$), C_{Growth} ($p = 0.0121$), C_{mic} ($p = 0.0080$), and SOC ($p = 0.0358$) increased significantly with ley farming as compared to the conventional crop rotation, but not $C_{Respiration}$. The given p-values indicate significant differences for the tested contrast ley farming versus crop rotation. Furthermore, a post hoc test revealed that CUE in ley farmed soils (emmean 0.34 ± SE 0.02, group: a) was more similar to grassland (emmean 0.39 ± SE 0.02, group: a) than cropland soils (emmean 0.28 ± SE 0.02, group: b).

3.2 | The Influence of Sampling Time on Microbial CUE

In accordance with the meta-analysis approach, we did not find any treatment effect on CUE, in the site-wise ANOVA, with exception of the ley farming that showed significantly higher CUE than the arable crop rotation ($p < 0.001$). The effect of treatment on CUE did not vary between the samplings, that is, there was no interaction between sampling and treatment. Most interestingly, we found that CUE differed significantly between samplings at all sites (Sweden: $p = 0.027$, the Netherlands, France, Slovenia: $p < 0.001$) except for Spain ($p = 0.124$) (Figure 4). Differences in CUE between samplings exceeded the mean differences between sites (Figure 4).

3.3 | Driver Analysis

Weather predictors explained 36% ($R^2_{marginal} = 0.36$; $R^2_{conditional} = NA$) of the variation in CUE data according to the linear mixed-effects model (Table S4). Site as a random factor, however, did not explain much of the residual variation, with a random effect estimate of 0.00 versus 0.64 residual effects (Table S4). Most interestingly, CUE was significantly lower in the warm season, that is, with higher Δ temperature ($p < 0.001$) (Figure 5). In line, rising temperatures prior to sampling had a negative effect on CUE ($p = 0.014$). Water content at sampling was not an important predictor of seasonal differences in CUE. However, when soil was sampled during a time of high precipitation (relative to MAP), associated CUE was higher ($p = 0.011$). High photosynthetically active radiation over 3 months prior sampling was associated with higher CUE as well ($p = 0.020$).

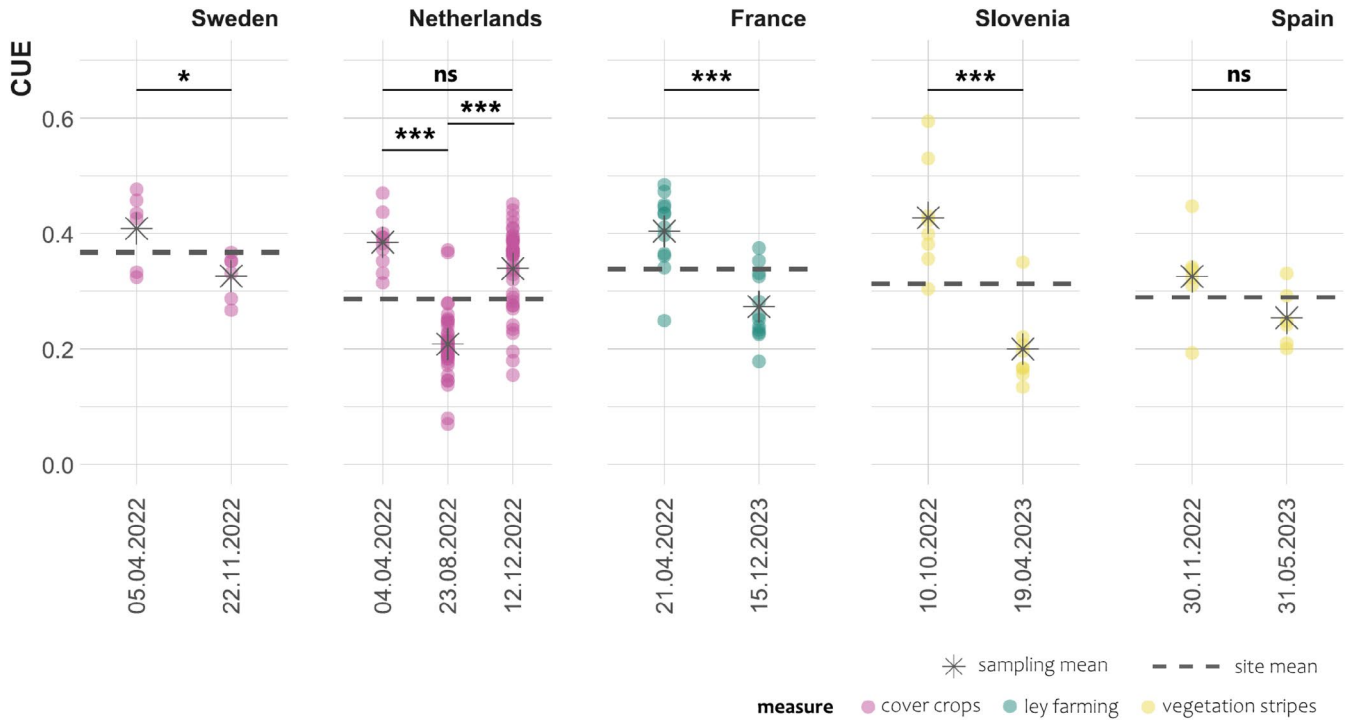

FIGURE 4 | Microbial carbon use efficiency (CUE) differs between sampling time points. Colours indicate the respective diversification measure of the established experiment. Asterisks mark significant differences between samplings (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

4 | Discussion

4.1 | Crop Diversification Has No General Effect on Microbial CUE and SOC Storage

Across the pedoclimatic European gradient, there was no general effect of crop diversification measures on microbial CUE, microbial activity or biomass. The effect of crop diversification on microbial C transformation differed, however, between different measures.

The introduction of vegetation stripes did not alter microbial physiology but increased its overall biomass and activity (Figure 3). In line with that, no change in CUE was observed in a study with diversification of grassland species in the Jena experiment (Germany) (Prommer et al. 2020), which comprises more than 80 plots (20 m × 20 m) with artificial grassland communities of 1, 2, 4, 8, 16, and 60 species. At the Jena experiment, species-rich plant communities promoted microbial growth and microbial biomass most likely through greater plant organic matter inputs (i.e., root biomass), according to a path analysis. The authors concluded that this effect accumulated over time (established in 2002) supporting SOC accumulation in this extensive hay meadow. Similarly, our results indicate that vegetation stripes support C accrual by either increasing C inputs as compared to bare fallow stripes between permanent crops, or preventing SOC losses through erosion. We observed that the introduction of a permanent vegetation stripe in Spain (13 years) and Slovenia (25 years) increased microbial biomass, activity and SOC (Figure 3). Given the absence of changes in CUE (Figure 3), increases in SOC with vegetation stripes may not be linked to changes in microbial physiology but rather to higher inputs and improved erosion protection.

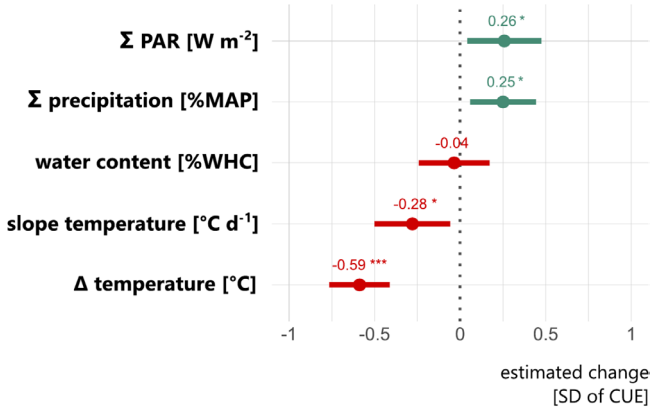

FIGURE 5 | Fixed effects of seasonal predictors on CUE in the linear mixed-effects model. Scaled estimates indicate how much of the variance in CUE is explained by a change of one standard deviation in the predictor variable (Σ PAR: 3-month cumulative photosynthetically active radiation; Σ precipitation: 3-month cumulative precipitation; slope temperature: Slope extracted from regression of mean daily temperature over day for 3 months prior sampling; Δ temperature: Difference between mean 3-month temperature and mean annual temperature). Asterisks mark significant fixed effects of predictor on CUE (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Also, cover cropping did not affect CUE (Figure 3). In line with our results, no effect of winter cover crops (i.e., oil radish following rye, ryegrass following potato) was observed on CUE for a German arable soil in two subsequent years, although aboveground diversification resulted in a more diverse microbial community (Liu et al. 2023). A higher Chao1 diversity was also reported in the diverse species mixtures of rhizosphere soils at the Austrian site (personal communication). There is little

indication that a more diverse microbial community through crop diversification supports higher CUE. The introduction of an additional species into a crop rotation, as practiced with cover crops, is limited to a short-period and effects could thus only occur during the time of established cover crops. However, we observed no significant difference between the effects of cover crops on CUE in the Swedish and Dutch sites, neither for the season when cover crops were grown nor when the main crops were present. This suggests that bulk soil CUE was not affected by cover crops. Despite their relatively short growing period, cover crops are generally considered a useful measure to promote SOC formation with reported change rates of +0.30 Mg C ha^{-1} $year^{-1}$ (Poeplau and Don 2015; Seitz et al. 2023). However, in this study, we did not observe a significant overall effect of cover crops on SOC content (Figure 3). This may be related to the young age of the field experiments in Lithuania, Czech Republic and Austria (i.e., 1, 2, and 3 years, respectively). Applying the reported accrual rates for cover crops (Poeplau and Don 2015; Seitz et al. 2023) and assuming a bulk density of 1.4 g kg^{-1}, SOC content would only increase by 0.007 g kg^{-1} soil $year^{-1}$. Changes in SOC would thus only be detectable after more than a decade. In our study, ryegrass cover tended to increase SOC after 39 years in the Swedish site, but the increase was not significant compared to control plots. However, the same ryegrass cover was previously reported to significantly increase SOC stocks at this experimental site when applied together with 90 kg ha^{-1} N fertilisation (Poeplau et al. 2015). Similar to the vegetation stripes, there was no evidence that cover crops altered microbial C transformation through changes in physiology. According to a meta-analysis on the effect of cover crops on SOC pools, cover crops increased mostly the particulate organic C (+ 23.2%) and microbial biomass C (+ 20.2%), while mineral-associated organic C showed a modest increase (+4.8%) (Fohrafellner et al. 2024). The latter C pool is generally assumed to increase by the in vivo pathway of C stabilisation under high CUE (Liang et al. 2017). The findings of Fohrafellner et al. (2024) indicate, similarly to our results, that effects of cover crops on bulk SOC are unlikely related to altered microbial physiology.

For the ley farming system at Lusignan (France), we found that crop diversification increased CUE by a factor of 1.21 (95% CI: 1.00–1.47) (Figure S2). Here, microbial biomass, C_{Growth}, and SOC, but not $C_{Respiration}$ increased with ley farming as compared to the conventional crop rotation. This suggests that the introduction of a 3-year grassland into crop rotation has altered microbial physiology or community composition to more efficient C use. Furthermore, we found that the CUE of ley farming was more similar to that of permanent grassland than that of cropland. Although the ley farming CUE tended to be lower, it was not significantly different from that of grassland soils, while the CUE of cropland was significantly lower than both ley farming and grassland CUE. CUE can differ strongly with land use type (e.g., Bölscher et al. 2016; He et al. 2023; Schroeder et al. 2024), with grassland microbial communities tending to have higher CUE values than cropland (He et al. 2023). There are few studies on microbial CUE with a focus on ley farming and the comparison to cropland. For a Swedish field experiment, no significant differences in substrate-specific CUE (i.e., Glycogen, Glucose, Alanine) were found between cropland, ley farming and grassland at incubation temperatures of ≤ 12.5°C using a thermodynamic efficiency approach to calculate CUE (Bölscher et al. 2016; Bölscher et al. 2020). However, at temperatures > 12.5°C the CUE of ley farming and grassland soils decreased, while that of cropland soils remained constant. Difference in the temperature-sensitivity of CUE between ley farming and grassland soils on one side, and cropland soils on the other side, may indicate that microbial C transformation in ley farming is more similar to grassland than to cropland (Bölscher et al. 2020). Indeed, the ley farming soil microbial community during the time of green fallow resembled the grassland community more than the cropland community (Bölscher et al. 2016). The introduction of temporal grassland into a crop rotation may therefore be considered rather a temporal land use change to grassland than just a crop diversification measure. In our study, samples were taken in the years of crop rotation subsequent 3 years of ley farming. The observed ley farming effect on CUE could therefore be considered as legacy of the grassland period. This legacy effect on CUE could possibly be linked to legacy effects on the microbial community (Jangid et al. 2011) or remaining dead roots from the ley farming period serving as substrate. However, we are limited in extrapolating our findings to other ley farming systems and more research is needed to investigate the effects of ley farming on microbial physiology and C transformation, and the question, why the introduction of permanent grassy vegetation stripes in permanent crop systems did not induce similar changes. Differences could potentially result from varying management strategies or climatic factors.

In summary, our results demonstrate that CUE and underlying parameters are not affected by aboveground plant diversification in agricultural soils per se. It is worth noting that we assessed potential CUE on disturbed bulk samples, while the effect of crop diversification likely induces most of the changes in the rhizosphere. Indeed, Domeignoz-Horta et al. (2024) report increased rhizosphere CUE with higher cover crop species richness (one to eight underseeded species) in a barley intercropping system, where rhizosphere soil is the soil attached to the roots of barley after excavating the plants and shaking them. It remains unknown whether the investigated crop diversification measures, which did not include intercropping, had effects on rhizosphere CUE. Such potential effects on rhizosphere CUE could have been diluted to undetectable levels in bulk samples. Despite this limitation, it can be concluded that crop diversification had little effect on SOC stocks, which was not mediated by the mechanism of altered microbial physiology at the bulk soil level.

4.2 | CUE Varies With Season

Most interestingly, CUE shifted significantly between the sampling events at four out of five sites (Figure 4). There is some indication that this observed variation with sampling could be related to seasonal dynamics in CUE. Previously, distinct seasonal patterns were reported for an arable and forest topsoil (0–5 cm): while overall activity was lowest during winter, CUE and microbial biomass C were highest (Schnecker et al. 2023). Likewise, significantly higher CUE values of approximately 0.45 were reported for a winter sampling (cold, wet) as compared to a summer sampling with CUE-values of approximately 0.20 (warm, dry) for a grassland topsoil (0–10 cm) (Ullah et al. 2021). To investigate seasonal effects on CUE, we did not assign

seasonal categories, which was not meaningful given the pedoclimatic gradient studied, but used a set of weather indicators as seasonal predictors. The aim of the driver analysis was to investigate whether there is a common seasonal pattern across sites. We observed warm and cold season, indicated by Δ temperature, to be the strongest predictor of seasonal variation (Figure 5). The CUE was lower for soils sampled during a period of higher temperatures. This is in line with Ullah et al. (2021), who also observed CUE to be lower during the warm and dry season, using the ^{18}O-labelling method at an incubation temperature of 20°C. Although Schnecker et al. (2023) also found a higher CUE (^{18}O-labelling method) in the cold season, it remains unknown to which extent the variation in incubation temperature may have affected their results given that CUE was determined at the respective field temperature at sampling. While the first approach allows assessing the potential CUE, the latter is more representative of the actual in situ conditions.

The fact that seasonal variation depended strongly on warm vs. cold season (i.e., Δ temperature) may also explain why we did not find significant differences between sampling occasions in Spain. At this site, both sampling events were conducted during the warm season according to the Δ temperature indicator. Furthermore, rising temperatures over the 3-month period before sampling, indicated by slope temperature, were associated with lower CUE (Figure 5). The influence of temperature on microbial CUE has gained a lot of attention and in laboratory studies, warming has yielded contrasting effects on the potential CUE during soil organic matter decomposition (i.e., ^{18}O-labelling method): some found the CUE to decrease (Li et al. 2021; Liu et al. 2021), being unaltered (Walker et al. 2018; Poeplau et al. 2019), or increased (Zheng et al. 2019; Schroeder et al. 2022). However, these studies altered incubation temperatures, while here, we assessed the CUE at 15°C and found it to depend on the environmental conditions during the 3 months prior sampling. The observed effect must thus be considered a legacy effect of the season, shaping for example the nutrients availability and the active microbial community. Results of Schnecker et al. (2023) suggest that CUE increases during the cold season may be related to an increase in microbial biomass C per unit of DNA, that is, *f*DNA. The authors hypothesised that microbes had produced storage compounds or cryoprotectants and by that increased their biomass. Indeed, the incorporation of storage compounds into microbial biomass has hardly been considered as a microbial growth pathway so far (Mason-Jones et al. 2023). The fact that the 3-month cumulative photosynthetically active radiation positively influenced CUE, suggests that seasonal dynamics of CUE also depend on plant activity, for example, root exudation. Given that substrate-induced CUE changes with the substrates (e.g., Bölscher et al. 2016), and roots exude various easily available substrate it is likely that microbial CUE responds to seasonal variations in root exudation. The current stage of plant development could therefore also induce variation in CUE. The 3-month cumulative precipitation also positively influenced CUE, even though water content at sampling had a minor importance for the seasonal variation in CUE. This is logical, since water content at sampling reflects only conditions within a very brief period prior to sampling, and is therefore not reflective of the period which may indirectly affect CUE via its effects on organic matter availability and the microbial community. The 3-month cumulative precipitation and water content at sampling did not take into account short-term fluctuations in rainfall patterns. Such variability in rainfall patterns can affect microbial community composition (Cregger et al. 2012), which can remain present in soil as a legacy (Meisner et al. 2021). The model was limited in explaining the variation in CUE between the sampling events ($R^2 = 0.36$), suggesting missing explanatory variables related for example to plant growth stage and management, for example, fertilisation or tillage. We have tried to identify general causes of CUE changes between samplings at the five sites, that is, if changes in CUE were rather related to changes in C_{Growth}, *f*DNA, or $C_{Respiration}$. However, there were no clear patterns and the mechanisms seemed to differ between sites. Our dataset did not inform on the underlying mechanisms behind the seasonal CUE variation. Further research is needed to study why microbial CUE may follow seasonal patterns as observed in this study.

The variation between sampling events blurred any potential differences between sites, raising the question of when to sample if site differences should be the focus of a study. It remains unclear how large-scale studies, investigating CUE of soil samples collected at different times (e.g., meta-analyses investigating global drivers of CUE) can be harmonised to account for large seasonal variation in CUE. Advantageously, according to the ANOVA the effect of treatment did not vary with sampling, suggesting that the time of sampling does not matter for the assessment of crop diversification effects on CUE. This was further supported by the low to moderate heterogeneity in effect size of crop diversification on CUE in the meta-analysis approach, that is, no differences in effect sizes between samplings.

In summary, our study pointed to temperature as an important predictor of seasonal variation in CUE. Seasonal patterns in the potential CUE may reflect the environmental conditions shaping the active microbial community and nutrient availability. Thus, climate predictors could be indirect indicators of the actual drivers of the variation in CUE with season. It remains unclear how informative a single measurement of CUE can be on microbial physiology affecting SOC dynamics, given that it will always reflect just a snapshot of a long-term process. However, driver analysis and treatment comparison are useful methods to gain understanding of these microbial processes.

5 | Conclusion

Our results imply that there is no general effect of crop diversification on potential CUE or other physiological parameters in bulk soil. We found no implication that crop diversification positively impacts C storage through altered microbial physiology. However, the potential of temporal grassland establishment to support SOC accrual through a legacy effect on microbial physiology needs further investigation. Additional C inputs from vegetation stripes can increase overall microbial biomass, activity and SOC likely due to higher C inputs. Thus, establishing vegetation stripes between permanent crops in the Mediterranean appears beneficial for C accrual. Moreover, CUE varied more with season than with treatment or site, highlighting the need to cover seasonal patterns of microbial activity and community dynamics to understand microbial C transformation and its link to soil C storage. In general, linking potential CUE (which reflects

short-term dynamics) to SOC changes (which occur over long-term) remains challenging.

Author Contributions

Julia Schroeder: conceptualization, investigation, data curation, formal analysis, visualization, writing – review and editing, writing – original draft. **Alexander König:** investigation, writing – review and editing. **Christopher Poeplau:** conceptualization, funding acquisition, writing – review and editing. **Tobias Bölscher:** conceptualization, funding acquisition, writing – review and editing. **Katharina H. E. Meurer:** conceptualization, funding acquisition, writing – review and editing, resources. **Monika Toleikienė:** resources. **Marjoleine Hanegraaf:** resources, conceptualization, funding acquisition. **Annelein Meisner:** conceptualization, funding acquisition, writing – review and editing. **Josef Hakl:** resources, writing – review and editing. **Katharina M. Keiblinger:** conceptualization, funding acquisition, writing – review and editing, resources. **Abad Chabbi:** conceptualization, funding acquisition, writing – review and editing, resources. **Marjetka Suhadolc:** conceptualization, funding acquisition, writing – review and editing, resources. **Anton Govednik:** investigation. **Erich Inselsbacher:** conceptualization, funding acquisition. **Heike Knicker:** conceptualization, funding acquisition, resources. **Laura Gismero Rodríguez:** investigation. **Anke M. Herrmann:** conceptualization, funding acquisition, project administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Zenodo (DOI: 10.5281/zenodo.13271731).

References

Aronsson, H., and G. Torstensson. 1998. "Measured and Simulated Availability and Leaching of Nitrogen Associated With Frequent Use of Catch Crops." *Soil Use and Management* 14: 6–13. https://doi.org/10.1111/j.1475-2743.1998.tb00603.x.

Blanchy, G., G. Bragato, C. Di Bene, et al. 2023. "Soil and Crop Management Practices and the Water Regulation Functions of Soils: A Qualitative Synthesis of Meta-Analyses Relevant to European Agriculture." *Soil* 9: 1–20. https://doi.org/10.5194/soil-9-1-2023.

Bölscher, T., G. I. Ågren, and A. M. Herrmann. 2020. "Land-Use Alters the Temperature Response of Microbial Carbon-Use Efficiency in Soils—A Consumption-Based Approach." *Soil Biology and Biochemistry* 140: 107639. https://doi.org/10.1016/j.soilbio.2019.107639.

Bölscher, T., L. Wadsö, G. Börjesson, and A. M. Herrmann. 2016. "Differences in Substrate Use Efficiency: Impacts of Microbial Community Composition, Land Use Management, and Substrate Complexity." *Biology and Fertility of Soils* 52: 547–559. https://doi.org/10.1007/s00374-016-1097-5.

Cregger, M. A., C. W. Schadt, N. G. McDowell, W. T. Pockman, and A. T. Classen. 2012. "Response of the Soil Microbial Community to Changes in Precipitation in a Semiarid Ecosystem." *Applied and Environmental Microbiology* 78: 8587–8594. https://doi.org/10.1128/AEM.02050-12.

De Graaff, M.-A., A. T. Classen, H. F. Castro, and C. W. Schadt. 2010. "Labile Soil Carbon Inputs Mediate the Soil Microbial Community Composition and Plant Residue Decomposition Rates." *New Phytologist* 188: 1055–1064. https://doi.org/10.1111/j.1469-8137.2010.03427.x.

Domeignoz-Horta, L., S. L. Cappelli, R. Shrestha, et al. 2024. "Plant Diversity Drives Positive Microbial Associations in the Rhizosphere Enhancing Carbon Use Efficiency in Agricultural Soils." *Nature Communications* 15: 8065. https://doi.org/10.1038/s41467-024-52449-5.

Duan, P., R. Fu, A. T. Nottingham, et al. 2023. "Tree Species Diversity Increases Soil Microbial Carbon Use Efficiency in a Subtropical Forest." *Global Change Biology* 29: 7131–7144. https://doi.org/10.1111/gcb.16971.

Elhakeem, A., R. J. Porre, E. Hoffland, J. C. van Dam, S. M. Drost, and G. B. de Deyn. 2023. "Radish-Based Cover Crop Mixtures Mitigate Leaching and Increase Availability of Nitrogen to the Cash Crop." *Field Crops Research* 292: 108803. https://doi.org/10.1016/j.fcr.2022.108803.

Eurostat. 2022. *Utilised Agricultural Area by Categories*. Eurostat.

Fendrich, A. N., F. Matthews, E. van Eynde, et al. 2023. "From Regional to Parcel Scale: A High-Resolution Map of Cover Crops Across Europe Combining Satellite Data With Statistical Surveys." *Science of the Total Environment* 873: 162300. https://doi.org/10.1016/j.scitotenv.2023.162300.

Fohrafellner, J., K. M. Keiblinger, S. Zechmeister-Boltenstern, R. Murugan, H. Spiegel, and E. Valkama. 2024. "Cover Crops Affect Pool Specific Soil Organic Carbon in Cropland—A Meta-Analysis." *European Journal of Soil Science* 75, no. 2: e13472. https://doi.org/10.1111/ejss.13472.

Fohrafellner, J., S. Zechmeister-Boltenstern, R. Murugan, and E. Valkama. 2023. "Quality Assessment of Meta-Analyses on Soil Organic Carbon." *Soil* 9: 117–140. https://doi.org/10.5194/soil-9-117-2023.

Geyer, K. M., P. Dijkstra, R. Sinsabaugh, and S. D. Frey. 2019. "Clarifying the Interpretation of Carbon Use Efficiency in Soil Through Methods Comparison." *Soil Biology and Biochemistry* 128: 79–88. https://doi.org/10.1016/j.soilbio.2018.09.036.

Gurevitch, J., and L. V. Hedges. 1999. "Statistical Issues in Ecological Meta-Analyses." *Ecology* 80: 1142. https://doi.org/10.2307/177061.

Hakl, J., E. Kunzová, Š. Tocauerová, L. Menšík, M. Mrázková, and J. Pozdíšek. 2021. "Impact of Long-Term Manure and Mineral Fertilization on Yield and Nutritive Value of Lucerne (*Medicago sativa*) in Relation to Changes in Canopy Structure." *European Journal of Agronomy* 123: 126219. https://doi.org/10.1016/j.eja.2020.126219.

Harrer, M., P. Cuijpers, T. A. Furukawa, and D. D. Ebert. 2022. *Doing Meta-Analysis With R: A Hands-On Guide*. 1st ed. CRC Press Taylor & Francis Group.

He, P., Y. Zhang, Q. Shen, N. Ling, and Z. Nan. 2023. "Microbial Carbon Use Efficiency in Different Ecosystems: A Meta-Analysis Based on a Biogeochemical Equilibrium Model." *Global Change Biology* 29: 4758–4774. https://doi.org/10.1111/gcb.16861.

Hedges, L. V., J. Gurevitch, and P. S. Curtis. 1999. "The Meta-Analysis of Response Rations in Experimental Ecology." *Ecology* 80: 1150–1156. https://doi.org/10.1890/0012-9658(1999)080[1150:TMAORR]2.0.CO;2.

Hu, T., and A. Chabbi. 2022. "Grassland Management and Integration During Crop Rotation Impact Soil Carbon Changes and Grass-Crop

Production." *Agriculture, Ecosystems & Environment* 324: 107703. https://doi.org/10.1016/j.agee.2021.107703.

Jangid, K., M. A. Williams, A. J. Franzluebbers, T. M. Schmidt, D. C. Coleman, and W. B. Whitman. 2011. "Land-Use History Has a Stronger Impact on Soil Microbial Community Composition Than Aboveground Vegetation and Soil Properties." *Soil Biology and Biochemistry* 43: 2184–2193. https://doi.org/10.1016/j.soilbio.2011.06.022.

Joergensen, R. G. 1996. "The Fumigation-Extraction Method to Estimate Soil Microbial Biomass: Calibration of the kEC Value." *Soil Biology and Biochemistry* 28: 25–31. https://doi.org/10.1016/0038-0717(95)00102-6.

Kallenbach, C. M., S. D. Frey, and A. S. Grandy. 2016. "Direct Evidence for Microbial-Derived Soil Organic Matter Formation and Its Ecophysiological Controls." *Nature Communications* 7: 13630. https://doi.org/10.1038/ncomms13630.

Karger, D. N., O. Conrad, J. Böhner, et al. 2017. "Climatologies at High Resolution for the Earth's Land Surface Areas." *Scientific Data* 4: 170122. https://doi.org/10.1038/sdata.2017.122.

Karger, D. N., O. Conrad, J. Böhner, et al. 2018. "Data From: Climatologies at High Resolution for the Earth's Land Surface Areas." Dryad.

Launay, C., J. Constantin, F. Chlebowski, et al. 2021. "Estimating the Carbon Storage Potential and Greenhouse Gas Emissions of French Arable Cropland Using High-Resolution Modeling." *Global Change Biology* 27: 1645–1661. https://doi.org/10.1111/gcb.15512.

Lehmann, J., C. M. Hansel, C. Kaiser, et al. 2020. "Persistence of Soil Organic Carbon Caused by Functional Complexity." *Nature Geoscience* 13: 529–534. https://doi.org/10.1038/s41561-020-0612-3.

Li, J., J. Pei, F. A. Dijkstra, M. Nie, and E. Pendall. 2021. "Microbial Carbon Use Efficiency, Biomass Residence Time and Temperature Sensitivity Across Ecosystems and Soil Depths." *Soil Biology and Biochemistry* 154: 108117. https://doi.org/10.1016/j.soilbio.2020.108117.

Liang, C., M. Kästner, and R. G. Joergensen. 2020. "Microbial Necromass on the Rise: The Growing Focus on Its Role in Soil Organic Matter Development." *Soil Biology and Biochemistry* 150: 108000. https://doi.org/10.1016/j.soilbio.2020.108000.

Liang, C., J. P. Schimel, and J. D. Jastrow. 2017. "The Importance of Anabolism in Microbial Control Over Soil Carbon Storage." *Nature Microbiology* 2: 17105. https://doi.org/10.1038/nmicrobiol.2017.105.

Liu, B., J. Schroeder, H. Ahnemann, C. Poeplau, and C. C. Tebbe. 2023. "Crop Diversification Improves the Diversity and Network Structure of the Prokaryotic Soil Microbiome at Conventional Nitrogen Fertilization." *Plant and Soil* 489: 259–276. https://doi.org/10.1007/s11104-023-06011-9.

Liu, X. J. A., G. Pold, L. A. Domeignoz-Horta, et al. 2021. "Soil Aggregate-Mediated Microbial Responses to Long-Term Warming." *Soil Biology and Biochemistry* 152: 108055. https://doi.org/10.1016/j.soilbio.2020.108055.

Malik, A. A., J. Puissant, K. M. Buckeridge, et al. 2018. "Land Use Driven Change in Soil pH Affects Microbial Carbon Cycling Processes." *Nature Communications* 9: 3591. https://doi.org/10.1038/s41467-018-05980-1.

Manzoni, S., P. Taylor, A. Richter, A. Porporato, and G. I. Ågren. 2012. "Environmental and Stoichiometric Controls on Microbial Carbon-Use Efficiency in Soils." *New Phytologist* 196: 79–91. https://doi.org/10.1111/j.1469-8137.2012.04225.x.

Mason-Jones, K., A. Breidenbach, J. Dyckmans, C. C. Banfield, and M. A. Dippold. 2023. "Intracellular Carbon Storage by Microorganisms Is an Overlooked Pathway of Biomass Growth." *Nature Communications* 14: 2240. https://doi.org/10.1038/s41467-023-37713-4.

Meisner, A., B. L. Snoek, J. Nesme, et al. 2021. "Soil Microbial Legacies Differ Following Drying-Rewetting and Freezing-Thawing Cycles." *ISME Journal* 15: 1207–1221. https://doi.org/10.1038/s41396-020-00844-3.

Messéan, A., L. Viguier, L. Paresys, et al. 2021. "Enabling Crop Diversification to Support Transitions Toward More Sustainable European Agrifood Systems." *Frontiers of Agricultural Science and Engineering* 8: 474–480. https://doi.org/10.15302/J-FASE-2021406.

Pellerin, S., L. Bamière, C. Launay, et al. 2019. Stocker du carbone dans les sols français quel potentiel au regard de l'objectif de 4 pour 1000 et à quel coût?: Synthèse du rapport d'étude.

Poeplau, C., H. Aronsson, Å. Myrbeck, and T. Kätterer. 2015. "Effect of Perennial Ryegrass Cover Crop on Soil Organic Carbon Stocks in Southern Sweden." *Geoderma Regional* 4: 126–133. https://doi.org/10.1016/j.geodrs.2015.01.004.

Poeplau, C., and A. Don. 2015. "Carbon Sequestration in Agricultural Soils via Cultivation of Cover Crops—A Meta-Analysis." *Agriculture, Ecosystems & Environment* 200: 33–41. https://doi.org/10.1016/j.agee.2014.10.024.

Poeplau, C., M. Helfrich, R. Dechow, et al. 2019. "Increased Microbial Anabolism Contributes to Soil Carbon Sequestration by Mineral Fertilization in Temperate Grasslands." *Soil Biology and Biochemistry* 130: 167–176. https://doi.org/10.1016/j.soilbio.2018.12.019.

Posit team. 2024. *RStudio: Integrated Development Environment for R*. Posit Software, PBC.

Prommer, J., T. W. N. Walker, W. Wanek, et al. 2020. "Increased Microbial Growth, Biomass, and Turnover Drive Soil Organic Carbon Accumulation at Higher Plant Diversity." *Global Change Biology* 26: 669–681. https://doi.org/10.1111/gcb.14777.

R Core Team. 2024. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing.

Schnecker, J., L. Baldaszti, P. Gündler, et al. 2023. "Seasonal Dynamics of Soil Microbial Growth, Respiration, Biomass, and Carbon Use Efficiency in Temperate Soils." *Geoderma* 440: 116693. https://doi.org/10.1016/j.geoderma.2023.116693.

Schroeder, J., L. Kammann, M. Helfrich, C. C. Tebbe, and C. Poeplau. 2021. "Impact of Common Sample Pre-Treatments on Key Soil Microbial Properties." *Soil Biology and Biochemistry* 160: 108321. https://doi.org/10.1016/j.soilbio.2021.108321.

Schroeder, J., T. Peplau, E. Gregorich, C. C. Tebbe, and C. Poeplau. 2022. "Unexpected Microbial Metabolic Responses to Elevated Temperatures and Nitrogen Addition in Subarctic Soils Under Different Land Uses." *Biogeochemistry* 160: 105–125. https://doi.org/10.1007/s10533-022-00943-7.

Schroeder, J., T. Peplau, F. Pennekamp, E. Gregorich, C. C. Tebbe, and C. Poeplau. 2024. "Deforestation for Agriculture Increases Microbial Carbon Use Efficiency in Subarctic Soils." *Biology and Fertility of Soils* 60: 17–34. https://doi.org/10.1007/s00374-022-01669-2.

Schwartz, E. 2007. "Characterization of Growing Microorganisms in Soil by Stable Isotope Probing With H 218 O." *Applied and Environmental Microbiology* 73: 2541–2546. https://doi.org/10.1128/AEM.02021-06.

Seitz, D., L. M. Fischer, R. Dechow, M. Wiesmeier, and A. Don. 2023. "The Potential of Cover Crops to Increase Soil Organic Carbon Storage in German Croplands." *Plant and Soil* 488: 157–173. https://doi.org/10.1007/s11104-022-05438-w.

Sinsabaugh, R. L., B. L. Turner, J. M. Talbot, et al. 2016. "Stoichiometry of Microbial Carbon Use Efficiency in Soils." *Ecological Monographs* 86: 172–189. https://doi.org/10.1890/15-2110.1.

Soares, M., and J. Rousk. 2019. "Microbial Growth and Carbon Use Efficiency in Soil: Links to Fungal-Bacterial Dominance, SOC-Quality and Stoichiometry." *Soil Biology and Biochemistry* 131: 195–205. https://doi.org/10.1016/j.soilbio.2019.01.010.

Soong, J. L., L. Fuchslueger, S. Marañon-Jimenez, et al. 2020. "Microbial Carbon Limitation: The Need for Integrating Microorganisms Into Our Understanding of Ecosystem Carbon Cycling." *Global Change Biology* 26: 1953–1961. https://doi.org/10.1111/gcb.14962.

Spohn, M., K. Klaus, W. Wanek, and A. Richter. 2016. "Microbial Carbon Use Efficiency and Biomass Turnover Times Depending on Soil Depth—Implications for Carbon Cycling." *Soil Biology and Biochemistry* 96: 74–81. https://doi.org/10.1016/j.soilbio.2016.01.016.

Ullah, M. R., Y. Carrillo, and F. A. Dijkstra. 2021. "Drought-Induced and Seasonal Variation in Carbon Use Efficiency Is Associated With Fungi:Bacteria Ratio and Enzyme Production in a Grassland Ecosystem." *Soil Biology and Biochemistry* 155: 108159. https://doi.org/10.1016/j.soilbio.2021.108159.

Vance, E. D., P. C. Brookes, and D. S. Jenkinson. 1987. "An Extraction Method for Measuring Soil Microbial Biomass C." *Soil Biology and Biochemistry* 19: 703–707. https://doi.org/10.1016/0038-0717(87)90052-6.

Walker, T. W. N., C. Kaiser, F. Strasser, et al. 2018. "Microbial Temperature Sensitivity and Biomass Change Explain Soil Carbon Loss With Warming." *Nature Climate Change* 8: 885–889. https://doi.org/10.1038/s41558-018-0259-x.

Zheng, Q., Y. Hu, S. Zhang, et al. 2019. "Growth Explains Microbial Carbon Use Efficiency Across Soils Differing in Land Use and Geology." *Soil Biology and Biochemistry* 128: 45–55. https://doi.org/10.1016/j.soilbio.2018.10.006.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.