Soil Biology and Biochemistry 160 (2021) 108321

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Impact of common sample pre-treatments on key soil microbial properties

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ARTICLE INFO

Keywords:
Carbon use efficiency
^{18}O-labelling
Drying & rewetting
Birch effect
Freezing & thawing
Pre-incubation

ABSTRACT

Pre-treatment of soil samples prior to analysis is acknowledged to affect microbial activity and community parameters, but recommendations are diverging and there are no best-practice or standardised laboratory protocols. For the microbial carbon use efficiency (CUE) as a ratio parameter of C allocated to anabolism over total metabolised C, the effects of sample pre-treatment are unpredictable because the underlying parameters of microbial metabolism may be affected differently. In treatment or land use comparisons, it is important to know whether soils are affected in a similar way by pre-treatment. Based on five different paired sites (each with cropland and forest), the effects of six common pre-treatments were evaluated on ^{18}O-CUE and associated parameters, i.e. respiration rate, soil microbial biomass C, total DNA extracted, and estimated abundances of fungi, bacteria and archaea. The six common pre-treatments were: (i) immediate analysis of field-fresh soil samples, and analysis following 14 d pre-incubation of soil samples that were (ii) field-fresh, (iii) air-dried, (iv) oven-dried (40 °C), (v) frozen at −20 °C and (vi) frozen *in-situ* (dry ice and subsequently liquid N_2). The experiment revealed two main findings. The first was that pre-incubation of 14 d had the most pronounced effect and reduced microbial respiration rate, growth rate and microbial biomass C by between 28% and 63% on average compared with field-fresh samples, suggesting an impact of the reduction in readily available substrates on microbial activity. The second finding was that even after 14 days of pre-incubation, drying & rewetting caused an increase in the microbial respiration rate in forest soil samples of 64 ± 53% (air-drying) and 86 ± 65% (oven-drying), known as the Birch effect. However, CUE as a ratio parameter was found to be unaffected by sample pre-treatment, with only minor changes after 14 d of pre-incubation. Pre-treatment (including pre-incubation) affected cropland and forest samples similarly, with one exception: the estimated abundance of fungi increased only with drying & rewetting in forest soils. This suggests that dried cropland soils could potentially yield appropriate community data, while forest soils respond to soil drying in a more complex way. It was concluded that CUE values can be derived from soil samples regardless of whether they were dried & rewetted, frozen & thawed, or stored fresh.

1. Introduction

As key players in the terrestrial carbon (C) cycle, soil microorganisms are of interest in climate change research (Singh et al., 2010; Cavicchioli et al., 2019). Microorganisms decompose organic matter to gain energy and molecular resources for their maintenance, growth and mobility. Carbon dioxide (CO_2) respired within the aerobic decomposition process evolves from soil and is lost to the atmosphere. To a certain extent, however, metabolised C is allocated to microbial biomass. Dead microbial biomass, i.e. necromass, is structural material for stable soil organic matter and thus sequesters C in soil (Miltner et al., 2012; Kallenbach et al., 2016; Soong et al., 2020). The soil microbial carbon use efficiency (CUE) is defined as the ratio of C allocated to the anabolism (biosynthesis of microbial products) to the total of metabolised C (Manzoni et al., 2012; Sinsabaugh et al., 2013; Spohn et al., 2016a). As such, it is a measure of how efficiently the soil microbial community allocates C to growth. A high CUE indicates good growth conditions. Microbes need at least 12% of the C taken up for maintenance. Thus, due to stoichiometric restrictions and enzyme kinetics, the maximum theoretically possible microbial CUE under optimal conditions is 0.88 (Gommers et al., 1988). The shift between anabolic and catabolic processes largely determines the fate of C input into the soil (Schimel and

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https://doi.org/10.1016/j.soilbio.2021.108321
Received 25 March 2021; Received in revised form 27 May 2021; Accepted 30 May 2021
Available online 1 June 2021

Schaeffer, 2012). Thus, CUE is a useful tool for investigating the impact of ecosystem changes, such as climate change or land-use change, on soil C dynamics.

Different methodological approaches to determine CUE have been compared and reviewed in detail by Manzoni et al. (2012), Sinsabaugh et al. (2013), Joergensen and Wichern (2018) and Geyer et al. (2019). Within these different approaches, the ^{18}O-labelling method has attracted increasing attention in the last few years. Thiele-Bruhn et al. (2020) recently proposed that it should be implemented as a standard method for assessing CUE in ISO standardisation. The ^{18}O-labelling method is a powerful tool for studying the CUE of soil organic matter because the labelling itself does not affect the soil microbial C metabolism (Mau et al., 2015; Geyer et al., 2019). It is based on the concept that ^{18}O-enriched water in soil solution is incorporated into newly constructed ds-DNA within the microbial growth process (Schwartz, 2007). Besides the parameters of soil microbial activity, the DNA extracted with this method can also be used to gain information about the composition and functional potentials of the soil microbiome (Spohn et al., 2016b; Poeplau et al., 2019). The ^{18}O-labelling method therefore has the potential to link the analysis of microbial activity to the analysis of community composition.

There are different soil sample preparation measures to analyse microbial parameters, including the analysis of fresh soil, rewetting of dried samples or thawing of frozen samples prior to analysis. Soil sample handling, storage time and length of pre-incubation prior to analysis, together referred to below as 'pre-treatment', have long been known to affect measured parameters of soil microbial activity and community (Bartlett and James, 1980; Petersen and Klug, 1994; Stenberg et al., 1998; Franzluebbers, 1999).

Probably the best-known impact of sample pre-treatment is what is known as the Birch effect (Birch, 1958), which is a pulse in mineralisation and respiration after rewetting of dry soils. Pre-treatment conditions may place soil microbial cells under stress. According to Fierer and Schimel (2003), the short-term increase in microbial mineralisation observed after rewetting can be explained by a flush of cytoplasmic solutes from cells destroyed by the osmotic shock of drying. Freezing treatments might also result in osmotic stress and cell death due to increasing salt concentrations with ice formation, and ice crystals themselves could destroy cells (Stenberg et al., 1998). More recent research has focused on the impact of pre-treatment on, for example, enzyme activity (Lee et al., 2007; Peoples and Koide, 2012), microbial community composition (Pesaro et al., 2003; Lee et al., 2007; Rubin et al., 2013; Cui et al., 2014), community-level physiological profiles (CLPP) (Goberna et al., 2005) and the temperature sensitivity of soil respiration (Meyer et al., 2019). However, to date there has been an absence of research on the effect of pre-treatment on ^{18}O-CUE and associated parameters.

The choice of adequate pre-treatment appears to be particularly important in the use of the ^{18}O-labelling method for both microbial activity as well as community analysis because the proposed soil sample pre-treatments differ for individual parameters.

In the studies cited, it appears that the analysis of fresh samples is best suited to investigating microbial activity, while freezing is preferred for soil DNA-based microbial community analyses (Rubin et al., 2013; Cui et al., 2014). Some suggest that drying & rewetting should generally be avoided (Bartlett and James, 1980; Lee et al., 2007; Peoples and Koide, 2012), while others conclude that dry storage may be acceptable for sample comparisons (Meyer et al., 2019). If longer storage is unavoidable, freezing & thawing is also preferred for the analysis of parameters of microbial activity (Stenberg et al., 1998; Goberna et al., 2005; Peoples and Koide, 2012). Nevertheless, there are also indications that irreversible changes can occur to microbiological soil characteristics after freezing & thawing, such as a reduction in extractable DNA and the abundance of archaea (Pesaro et al., 2003), resulting in contradictory recommendations. Although a great deal of effort has been made to improve best-practice recommendations (Rhymes et al., 2020), the picture remains unclear. Pre-treatment can profoundly affect analytical results and no procedure is equally suitable for all analyses.

The questions arise of how sample pre-treatment affects CUE and the range of associated parameters of microbial metabolism derived by the ^{18}O-labelling method, and which pre-treatment is most suitable, especially if combined with an analysis of metagenomics.

Cost, time and logistics, as well as the availability of sample material, often outweigh any bias in data due to pre-treatment. Fresh soil is more difficult to handle, e.g. it is laborious to sieve. The freezing of soil needs storage capacity in freezers, which is energy intensive. Dry soil is biologically inactive and is therefore easier to store or transport, especially from remote areas. Thus, archive samples are often stored dry. Overall, the choice of pre-treatment is frequently an exercise in compromise. More information about the effects of pre-treatment on CUE estimates is needed to evaluate the potential of archived samples for research on soil microbial C dynamics.

A large number of biogeochemical studies focus on relative changes between land use types or management practices rather than absolute changes (Spohn et al., 2016b; Poeplau et al., 2019). However, some substantial differences in soil characteristics between land use types suggest that land use types may be altered differently by pre-treatment. Microbial community and dynamics are ecosystem specific. For example, forest soils are dominated by fungi much more than agricultural systems. Furthermore, the level and quality of soil organic carbon (SOC) stocks, which are land-use dependent, might influence the microbial metabolism. It is almost impossible and not always necessary to avoid biases in soil sampling, handling and analysis. For comparative studies, it is essential that the treatments are biased in a similar way (Peoples and Koide, 2012) otherwise treatment effects might be overestimated or underestimated. Standardised pre-treatments should be equally applicable to different systems. Literature on the comparability of pre-treatment effects between different treatments is scarce (e.g. Peoples and Koide, 2012; Meyer et al., 2019) and there is a lack of information about potential treatments or the land use dependencies of pre-treatment effects on ^{18}O-CUE. This study therefore also investigated whether pre-treatment-induced biases between treatments were proportional, in this case between cropland and forest.

This study investigated: i) how different sample pre-treatments (freezing & thawing, drying & rewetting, and fresh storage) affect CUE analysis and associated parameters, i.e. respiration rate, soil microbial biomass C, total DNA extracted, and estimated abundances based on marker gene copy numbers of fungi, bacteria and archaea, and ii) whether the soils of two contrasting land use types (cropland and forest) are affected in a similar way.

2. Material & methods

The effects of six common soil sample pre-treatments on CUE and associated parameters (respiration, soil microbial biomass C, growth and turnover, amount of DNA and estimated abundances of fungi, bacteria and archaea) on cropland and forest soils were evaluated using the ^{18}O-labelling method (Spohn et al., 2016a).

2.1. Sampling sites and sampling

Five paired sites (each with cropland and forest) near Braunschweig (Lower Saxony, Germany) were chosen in order to cover a range of different soil types. The sites are Bortfeld (*Bo*) (52°17′02.3″N 10°25′10.2″E), Harsum (*Ha*) (52°12′03.2″N 9°59′46.3″E), Hondelage (*Hl*) (52°19′49.4″N 10°36′23.4″E), Meine (*Me*) (52°23′17.4″N 10°31′25.5″E) and Völkenrode (*Vr*) (52°17′59.3″N 10°26′07.4″E). The soils are classified as Cambisols (*Bo, Hl, Vr*), Chernozem (*Ha*) and Podzol (*Me*) according to the World Reference Base for Soil Resources (IUSS Working Group WRB, 2015). The site characteristics are given in Table 1. The plots were located at least 10 m away from the edges of the respective land use type to avoid any disturbance effects. The croplands

Table 1
General soil parameters for all five paired sites: Bortfeld (*Bo*), Harsum (*Ha*), Hondelage (*Hl*), Meine (*Me*), Völkenrode (*Vr*).

Site	Land use	Texture			pH		C_{org} (%)	N_{total} (%)	C:N ratio	WHC (% w/w)
		sand (%)	silt (%)	clay (%)	H_2O (1:5 w/v)	$CaCl_2$ (1:5 w/v)				mean ± SD
Bo	forest	53	42	5	3.9	3.5	1.66	0.12	14	30 ± 3
Bo	cropland	60	34	6	7.0	6.5	1.23	0.13	9	29 ± 3
Ha	forest	2	77	21	5.3	5.0	4.33	0.33	13	66 ± 5
Ha	cropland	3	76	21	7.1	6.5	2.27	0.21	11	45 ± 5
Hl	forest	41	28	31	4.1	3.7	2.42	0.18	14	57 ± 3
Hl	cropland	36	27	37	7.0	6.4	1.90	0.17	11	47 ± 21
Me	forest	93	5	3	3.6	2.9	6.41	0.25	26	46 ± 4
Me	cropland	83	9	8	7.0	6.4	1.86	0.15	13	31 ± 1
Vr	forest	66	27	7	4.4	4.0	1.37	0.10	14	26 ± 0
Vr	cropland	67	27	6	4.7	4.4	0.77	0.05	15	19 ± 1

were bare on the date of sampling in March 2019. *Me* was the only coniferous forest site, while the other forests were deciduous.

Within an area 5 m × 5 m, a composite sample of five cuts was taken with a Dutch auger from the top 10 cm of mineral soil, then homogenised, divided into six and handled further according to the pre-treatment. In forests, the litter layer was removed manually before sampling. To obtain a field reference of the microbial community, an extra composite sample of 50 g was directly frozen on solid CO_2 and then frozen in liquid N_2 immediately after arriving in the laboratory, before being stored at −80 °C.

2.2. General soil parameters

General soil parameters, i.e. pH, C:N ratio, water content, water-holding capacity (WHC) and soil texture, were determined on 2-mm sieved oven-dried samples. Soil pH was measured using a pH glass electrode in a 1:5 w/v ratio of soil to 0.01 M $CaCl_2$ solution and H_2O, respectively, after shaking for 1 h on a horizontal shaker at 180 rpm. C and N contents were analysed by dry combustion (LECO TruMac, St. Joseph, MI, USA). Samples with $pH_{H2O} > 6.5$ were also analysed for carbonates using stepwise combustion at 450 °C for 12 h (LECO RC612, St. Joseph, MI, USA). Soil organic C (C_{org}) was calculated as the difference between total C and total inorganic C. Water content was assessed by drying samples to a constant mass at 105 °C. To quantify WHC, approximately 10 g oven-dried soil was placed on a funnel padded with cotton wool and soaked with H_2O by adding water using a wash bottle. Excess water was allowed to run off into a collecting vessel. The state of 100% WHC was assumed when no water accumulated on the soil surface and the water stopped dripping. About 5 g of the water-holding soil was dried to weight constancy at 105 °C. WHC was calculated as the percentage of water in the water-holding soil. Soil texture was measured according to DIN ISO 11277 (clay < 2 µm, silt 2–63 µm, sand >63 and < 2000 µm).

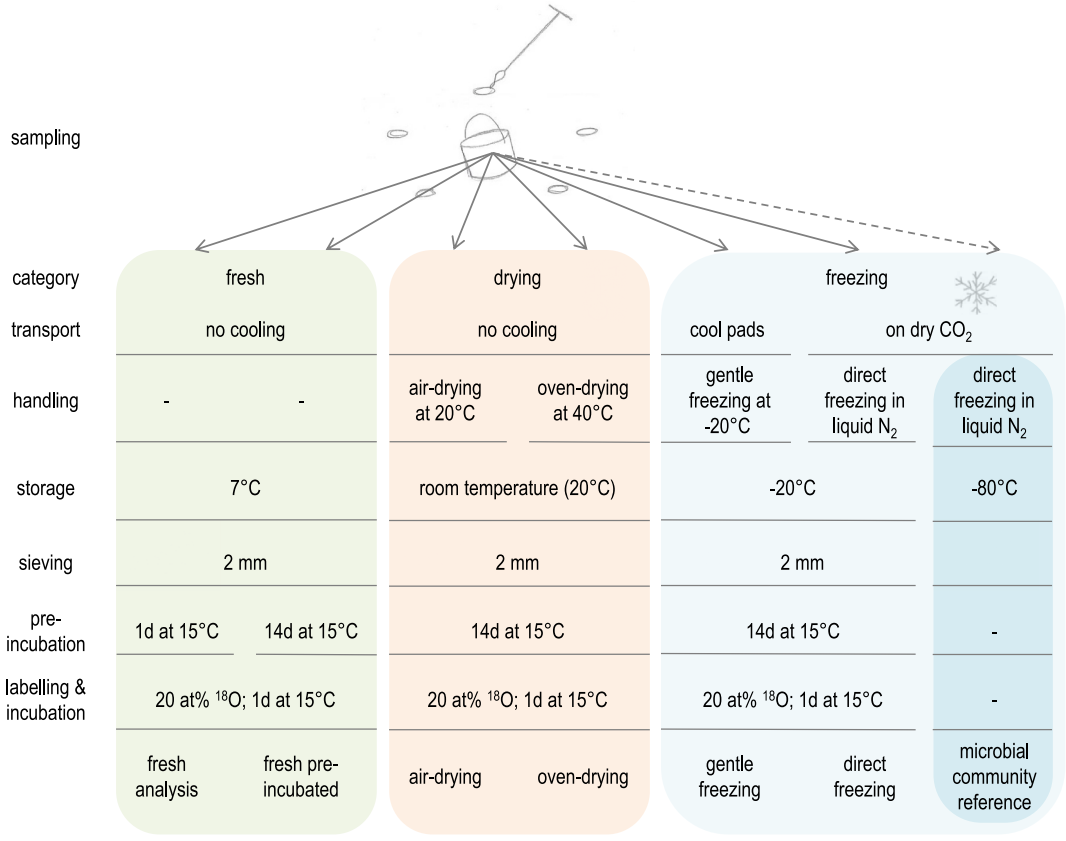

Fig. 1. Soil sample handling comprises the treatment of soil from transportation to pre-incubation. The experimental set-up involved six different pre-treatments, each with two variations of the main categories of fresh, drying and freezing. A microbial community reference was also sampled.

2.3. Experimental set-up

Soil sample pre-treatments can be divided into three main categories of processing: i) fresh, ii) dried and iii) frozen soil samples (Fig. 1). Sub-categories of soil sample pre-treatment were also defined, according to common practices described in the literature (Sheppard and Addison, 2008; Chen et al., 2010). The samples were handled in accordance with their particular pre-treatment, starting with transportation.

Fresh samples were transported in a box without further cooling since the outdoor temperature was around 10 °C on the day of sampling. Subsequently, samples were stored at 7 °C until 2-mm-sieving, water-content adjustment and pre-incubation. To quantify the effects of pre-incubation, the pre-treatments of fresh analysis and fresh pre-incubated differed in the duration of their pre-incubation of 1 d and 14 d at 15 °C respectively.

Within the category of dried soil samples, a differentiation was made between air-drying at room temperature and oven-drying at 40 °C. Samples were spread out in aluminium boxes directly after sampling and dried for approximately 96 h and 48 h in the air-drying and oven-drying pre-treatments respectively, under the conditions indicated, until no further mass reduction was observed. The resulting mean water content (mean ± SD, n = 10) was 2.87 ± 1.36 %WHC for air-dried samples and 1.95 ± 0.66 %WHC for oven-dried samples. Sieved dry samples were stored at room temperature until they were processed, which took place within five weeks.

Gentle freezing comprised transportation of soil samples with cool pads and freezing at −20 °C, while direct freezing involved transportation on dry CO_2 (ICEBITZZZ®, Linde AG) and freezing in liquid N_2 directly after arriving in the laboratory. Both freezing pre-treatments were stored at −20 °C and only thawed on the day of sieving, water-content adjustment and pre-incubation.

The sieving of fresh and frozen samples was time-consuming and laborious, while the sieving of dried soil samples was relatively quick and easy, with the exception of the clay-rich *Hl* site, which had to be pestled before it would pass through the 2-mm sieve.

For pre-incubation, three 20 g replicates of 2-mm-sieved soil per pre-treatment were filled into falcon tubes. Soil water content of fresh pre-incubated as well as dried and frozen samples was adjusted to 45 % WHC, which is 45% of the soils absolute WHC assessed on oven-dried soil of each individual plot. Water content was adjusted by adding water from a wash bottle by gravimetrical control. To ensure even rewetting, the air-dried and oven-dried samples were put into aluminium boxes prior to water addition and carefully mixed with a spoon before being transferred to falcon tubes. The falcon tubes were closed with a paper plug to enable gas exchange without microbial contamination. Except for fresh analysis, all the pre-treatments were incubated for 14 d at 15 °C. Water content of fresh analysis soils ranged from 31 %WHC (*Me* forest) to 56 %WHC (*TI* cropland) and was not adjusted for the 1 d of pre-incubation to avoid further disturbance. Notice that after 1 d or 14 d of pre-incubation the water content of all samples was adjusted to 60 %WHC within the ^{18}O-labelling method, so that during the 24 h of incubation soil moisture was equal for all pre-treatments.

Fresh analysis samples from all the sites were incubated in the week of sampling to keep the storage time of this pre-treatment to a minimum. Samples were incubated site-wise to avoid a bias within the pre-treatments due to different storage durations, i.e. fresh pre-incubated, air-dried, oven-dried, gently frozen, and directly frozen samples from one site were incubated at the same time. The day before incubation, water content was quantified to determine the actual dry-weight to wet-weight ratio (DW/WW) after pre-incubation.

2.4. Determination of ^{18}O-CUE

The ^{18}O-labelling method was conducted according to Spohn et al. (2016a) with the same modifications as described in Poeplau et al. (2019). Microbial biomass C was assessed by the chloroform-fumigation extraction (CFE) method (Vance et al., 1987). Fumigation was conducted for 24 h at room temperature in the dark and started the same day as the incubation, while non-fumigated samples were already extracted that day. Three and four extractant blanks were added for non-fumigated and fumigated extraction, respectively. Non-fumigated and fumigated aliquots of 7 g DW soil were extracted with 28 ml 0.5 M K_2SO_4 solution (1:4 w/v ratio) by horizontal shaking (180 rpm, 30 min), then filtered (hw3, Sartorius Stedim Biotech) and the extracts stored at 4 °C until measurement. Total C and total inorganic C were analysed in a 1:10 v/v extract dilution with double distilled water (Dimatoc, 2000; DIMATEC Analysentechnik GmbH). Total organic C was calculated as the difference between total C and total inorganic C. Microbial biomass C was calculated using a conversion factor of 0.45 (Joergensen, 1996).

For determination of microbial growth by incorporation of ^{18}O into the DNA, two aliquots of 300 mg WW soil were weighed into Eppendorf vials. The Eppendorf vials were placed in 20 ml glass vials and crimp-sealed. Based on the actual DW/WW ratio, the amount of labelled water needed to reach a labelling of 20 at% ^{18}O in the final soil water was calculated while adjusting the water content to 60 %WHC. Half of the samples were labelled with $H_2^{18}O$ (80 at% ^{18}O, diluted from 97 at% ^{18}O) with a syringe (Hamilton), while the same volume of double distilled water was added to the other half, serving as natural abundance controls. Vials with labelled samples were evacuated and flushed with standard gas of known CO_2 concentration (349 ppm) to a pressure of 1.3 bar immediately (within 1 min) after ^{18}O-water addition to equalise the starting conditions. Three gas blanks were added. Samples were incubated for 24 h at 15 °C in the dark. A headspace gas sample of 20 ml was taken from the labelled samples 24 h after labelling with a manual gas syringe (SGE Syringe, Trajan Scientific and Medical). The vials were de-crimped and soil samples immediately frozen in liquid N_2. Non-labelled and labelled samples were stored at −80 °C until DNA extraction.

Gas samples of the 24 h incubation were analysed for CO_2 concentration using a gas chromatograph equipped with an electron capture detector (Agilent 7890A GC, Agilent Technologies). The total amount of respired CO_2–C was calculated according to the ideal gas equation and related to g soil DW and time to obtain the respiration flux $C_{Respiration}$ [ng C g^{-1} soil DW h^{-1}]:

$$C_{Respiration} = \frac{p \times V}{R \times T} \times M \times \Delta CO_2 \times \frac{1}{\text{g soil} \times t} \quad [1]$$

where p is the pressure [kPa] in the vial (1300 kPa), V is the volume [l] of the vial headspace (22 ml), R is the universal gas constant (8314 J mol^{-1} K^{-1}), T is the temperature [K] at which the standard gas is injected into the vial (20 °C = 293.15 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 t [h].

DNA was extracted from labelled and non-labelled soil samples 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 4-16 KS). DNA was eluted in 100 µl DNase-free water and DNA concentration in the extracts was quantified with the QuantiT PicoGreen dsDNA Kit (Invitrogen). Then 60 µl of DNA eluate were transferred to silver capsules and oven-dried at 60 °C for 12 h. Isotopic analysis of ^{18}O in the dried DNA was 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). The amount of DNA produced within the incubation time was calculated according to Spohn et al. (2016a):

$$DNA_{produced} = DNA\ O \times \frac{DNA^{18}\ O}{enrichment} \times \frac{100}{31.21} \quad [2]$$

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 non-labelled 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 allocation rate to microbial biomass production C_{Growth} [ng C g^{-1} soil DW h^{-1}] is derived by transforming the amount of $DNA_{produced}$ into microbial biomass C produced, and related to g soil DW and time:

$$C_{Growth} = \frac{DNA_{produced} \times fDNA}{\text{g soil} \times t} \quad [3]$$

where the conversion factor *fDNA* is calculated for each individual sample as C_{mic} [µg g^{-1} soil DW] derived from the CFE analysis over total DNA [µg g^{-1} soil DW] derived from the PicoGreen screening.

The microbial CUE is defined as microbial biomass C produced over the total uptake of C, as an approximation of the sum of microbial biomass C produced and C respired (Manzoni et al., 2012; Sinsabaugh et al., 2013). The CUE value has no entity:

$$CUE = \frac{C_{Growth}}{C_{Growth} + C_{Respiration}} \quad [4]$$

Mass specific growth rate [d^{-1}] was calculated as C_{growth} [ng C g^{-1} soil DW h^{-1}] over C_{mic} [µg g^{-1} soil DW], which can be cancelled down to the following equation:

$$\text{mass specific growth rate} = \frac{DNA_{produced} \times \frac{24\ h}{t}}{\text{total DNA}} \quad [5]$$

2.5. Estimating microbial abundance by qPCR

The abundances of bacteria, archaea and fungi were estimated from the non-labelled DNA extracts by qPCR using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories) according to Hemkemeyer et al. (2015). In brief, the Taqman-probe approach was used for archaea and bacteria using the following primers for amplification of the 16S rRNA gene of archaea and bacteria respectively: ARC787F, ARC1059R, and BAC338F, BAC805R. And the probes ARC915F and BAC516F were used for quantification of the same gene, respectively (Yu et al., 2005). Fungal ITS1 sequences were amplified using the primers NSI1 and 58A2R and quantified by SYBR Green (Martin and Rygiewicz, 2005). Reactions were carried out in duplicates from 50 × and 100 × dilutions of the DNA extracts. Standard curves for the respective domains were generated using DNA from pure cultures of *Bacillus subtilis*, *Methanobacterium oryzae* and *Fusarium culmorum*. The PCR efficiencies were 98.1 ± 1.50% ($R^2 = 1.000$) for archaea, 96.0 ± 1.11% ($R^2 = 1.000$) for bacteria, and 89.6 ± 1.47% ($R^2 = 1.000$) for fungi.

2.6. Statistical analysis

All statistical analyses as well as data visualisation were conducted in R v3.6.3 (R Core Team, 2020) using RStudio v1.1.463 (RStudio Team, 2016) using the packages of the *tidyverse* (Wickham et al., 2019), *lme4* (Bates et al., 2015), *lmerTest* (Kuznetsova et al., 2017), *emmeans* (Lenth, 2021), *multcomp* (Hothorn et al., 2008), *multcompView* (Graves et al., 2019), and *cowplot* (Wilke, 2020). The randomized block design with land use as split-plots was addressed using a linear mixed effect model approach (lmer, *lme4*) with land use and pre-treatment as fixed effects and site as random effect, allowing for random intercept. A visual inspection of residual plots was used to check for deviations from homoscedasticity or normality and data was log-transformed were necessary (Table S3). Significance of fixed effects and their interaction were tested on the fitted model at a significance level of $\alpha = 0.05$. Estimated marginal means (emmeans, *emmeans*) were calculated for pre-treatments grouped by land use and differences between pre-treatments within a land use are given as compact-letter display. The *p*-values were adjusted according to Tukey. The relationships between C_{mic} and DNA extracted, K_2SO_4-extractable C and $C_{Respiration}$ or mass specific growth rate were tested for correlation using the Pearson correlation coefficient *r* (cor, *stats*).

All necessary raw data as well as the R code used to conduct statistical analysis and create figures are publicly accessible [DOI 10.5281/zenodo.4805813]. This study focused on the impact of pre-treatment effects on different parameters derived by the ^{18}O-labelling method, with sites serving as the replicate. Site differences were not of interest in relation to the present study's research question. Thus, site-specific and overall site means and standard deviations per land use of the individual parameters are provided in Tables S1 and S2 in the supplementary material.

3. Results

3.1. Parameters of soil microbial metabolism

Two main effects of pre-treatment were observed on the parameters associated with CUE. First, the most pronounced effect was a general reduction in the microbial parameters of metabolism with pre-incubation by on average 28% ($C_{Respiration}$, C_{mic}) to 63% (C_{Growth}). Second, drying & rewetting of soil samples led to an increase in $C_{Respiration}$, mass specific growth rate, C_{Growth} and K_2SO_4-extractable C in comparison with the fresh pre-incubated soil of on average 50% ($C_{Respiration}$, K_2SO_4-extractable C) to 132% (mass specific growth rate, C_{Growth}). The effect of drying was stronger across all parameters when the soils were oven-dried at 40 °C rather than air-dried.

Pre-incubation reduced $C_{Respiration}$ markedly in cropland soils by 30% ± 28% (mean ± SD) and in forest soils by 31% ± 45% indicated as the mean relative difference between fresh analysis and fresh pre-incubated (Fig. 2A). This reduction in $C_{Respiration}$ is also true for frozen pre-treatments. However, sample drying & rewetting before pre-incubation tended to increase $C_{Respiration}$ compared with fresh pre-incubated soils. In forest soils, the increases in $C_{Respiration}$ of 86% ± 65% (oven-drying) and 64% ± 53% (air-drying) were significant, however $C_{Respiration}$ in dried cropland soils was increased but not significantly different from fresh pre-incubated soil. Pre-treatment ($p < 0.001$) and land use ($p = 0.0057$) significantly affected $C_{Respiration}$. Both land use types were similarly affected by pre-treatment (Table S3).

Pre-incubation of 14 d reduced C_{mic} in fresh-preincubated soils compared to fresh analysis by approximately 7.5% ± 35% and 47% ± 26% in cropland and forest soils respectively. However, the reducing effect of 14 d pre-incubation on C_{mic}, as seen from the comparison between fresh analysis and fresh pre-incubated, was only significant in forest soils (Fig. 2B). The responses of forest and cropland soils to pre-incubation were significantly different ($p = 0.0366$). There was no indication for effects of soil sample freezing or drying on C_{mic} beyond the effect of pre-incubation, as there were no significant differences in C_{mic} between the frozen or dried and fresh pre-incubated soils (Fig. 2B). Land use ($p < 0.001$) and pre-treatment ($p < 0.001$) had significant effects on the mass specific growth rate (Table S3). Mass specific growth rate was significantly increased in dried soil samples in comparison with fresh pre-incubated samples, which showed lowest overall mass specific growth rate (Fig. 2C), without any differences between land use types.

The amounts of extractable C in the non-fumigated and fumigated K_2SO_4 extracts were significantly affected by pre-treatment ($p < 0.001$), i.e. pre-incubation reduced the amount of extractable C, while air-drying and oven-drying increased it (Fig. S1). The extractable C of non-fumigated soil is often used as a proxy for the labile C pool (Jones and Willett, 2006; Rousk and Jones, 2010). There was a significant positive correlation between K_2SO_4-extractable C and $C_{Respiration}$ ($r(178) = 0.15$, $p < 0.001$) and between K_2SO_4-extractable C and mass specific growth rate ($r(178) = 0.028$, $p = 0.02504$).

3.2. Microbial carbon use efficiency

Average CUE values for all sites were 0.24 ± 0.14 and 0.45 ± 0.26 in

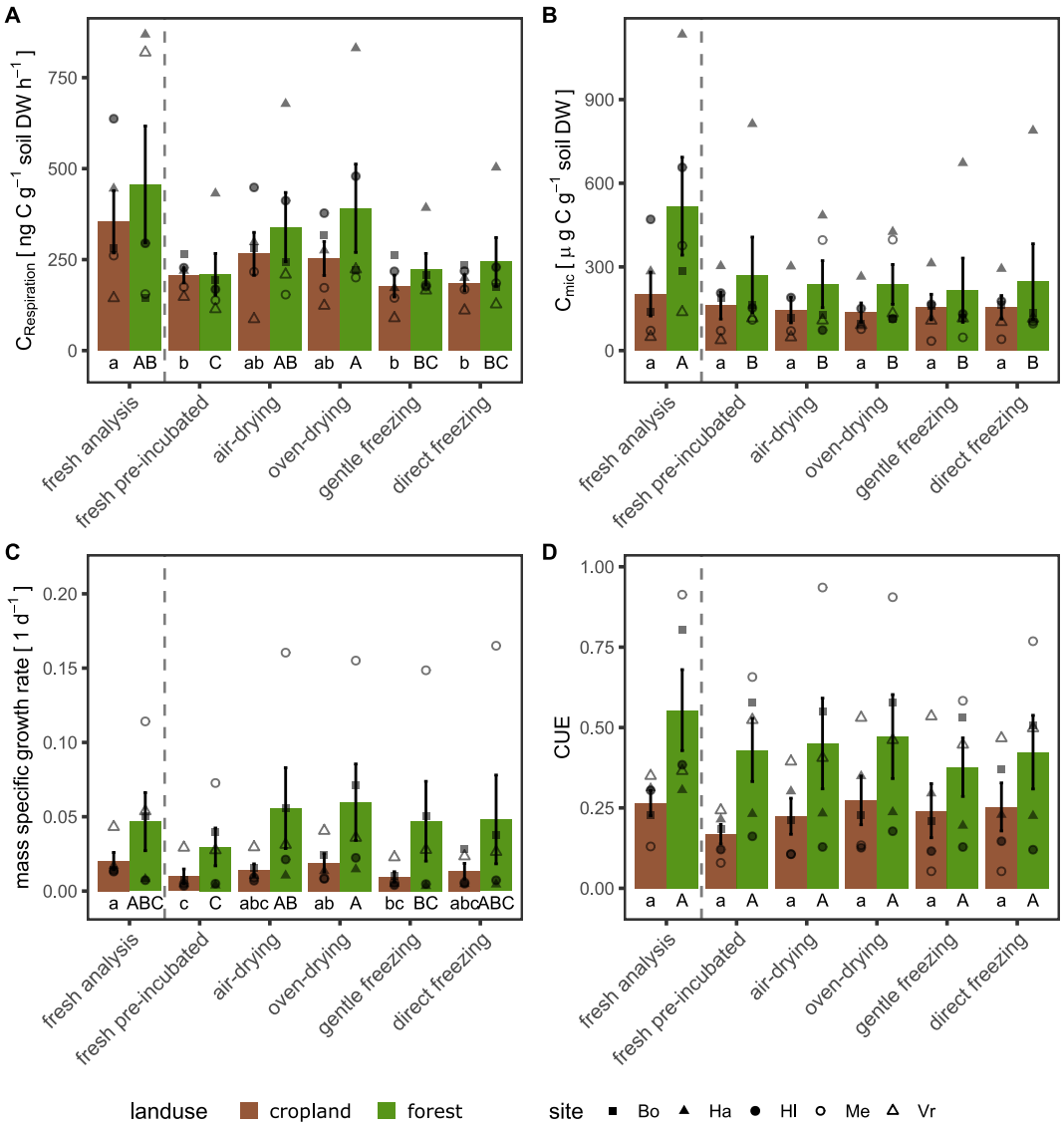

Fig. 2. Effect of soil sample pre-treatment on parameters of soil microbial metabolism: **A)** respiration rate ($C_{Respiration}$), **B)** microbial biomass C (C_{mic}) as derived by chloroform-fumigation extraction (CFE), **C)** mass specific growth rate, and **D)** microbial carbon use efficiency (CUE) derived by the ^{18}O-labelling method for the different pre-treatments according to land use. The bars indicate the overall mean and error bars give the standard error per pre-treatment (sites, n = 5) according to land use. Letters below the bars indicate significant differences according to estimated marginal means ($\alpha < 0.05$) between pre-treatments within the same land use type. Differently shaped points represent site means (replicates, n = 3). Statistical analyses on $C_{Respiration}$, C_{mic} and mass specific growth rate were conducted on log-transformed data.

cropland and forest respectively. Within the five sites, CUE values ranged from 0.03 to 0.63 in cropland soils and from 0.09 to 0.96 in forest soils. CUE values above the supposed stoichiometric maximum value of 0.88 (Gommers et al., 1988) were calculated for forest samples from the *Me* site: *Me* forest fresh analysis 0.91 ± 0.03, and *Me* forest air-drying and oven-drying 0.94 ± 0.03 and 0.91 ± 0.06 respectively. The *Me* site showed the greatest divergence in mean CUE between cropland (0.09 ± 0.05) and forest soils (0.79 ± 0.18). *Me* forest soil had a high C_{org} content (6.4%) and low pH_{CaCl2} = 2.9, while *Me* cropland showed a lower C_{org} content (1.9%) and much higher pH_{CaCl2} = 6.4, which might to some extent explain the wide range of CUE values between the land uses at *Me* site. While land use had a significant effect on CUE ($p < 0.001$), there was no evidence that CUE was affected by pre-treatment. Nonetheless, the comparison of fresh analysis with fresh pre-incubated samples depicts slight reductions in CUE with a 14 d long pre-incubation. Fresh pre-incubated showed a reduction of 36% ± 16% and 18% ± 37% in CUE compared with the fresh analysis samples in cropland and forest respectively.

The ^{18}O-labelling method uses the correlation of C_{mic} and total DNA extracted (*fDNA*) to convert the amount of DNA increase into newly produced microbial biomass C. As expected, a significant positive correlation was found between C_{mic} and the total DNA extracted for all pre-treatments in both land use types, except for oven-drying (Table S4). While there was only a small variation in the regression lines of the pre-incubated pre-treatments, fresh analysis clearly stood out, with lower extracted DNA per determined C_{mic} resulting in steeper regression lines (Fig. 3). Microbial biomass C was reduced by pre-incubation (Fig. 2A), while the amount of total DNA extracted was unaffected (Fig. S2). Correspondingly, the statistical analysis of total DNA extracted data revealed no significant differences between the pre-treatments or land use types (Table S3).

To understand the effect of drying and freezing on parameters associated with the ^{18}O-labelling method beyond the effect of pre-incubation time, which was the most pronounced effect, fresh pre-incubated was chosen to serve as the reference for microbial activity as it had undergone equal time of pre-incubation (Fig. 4, Figure S5).

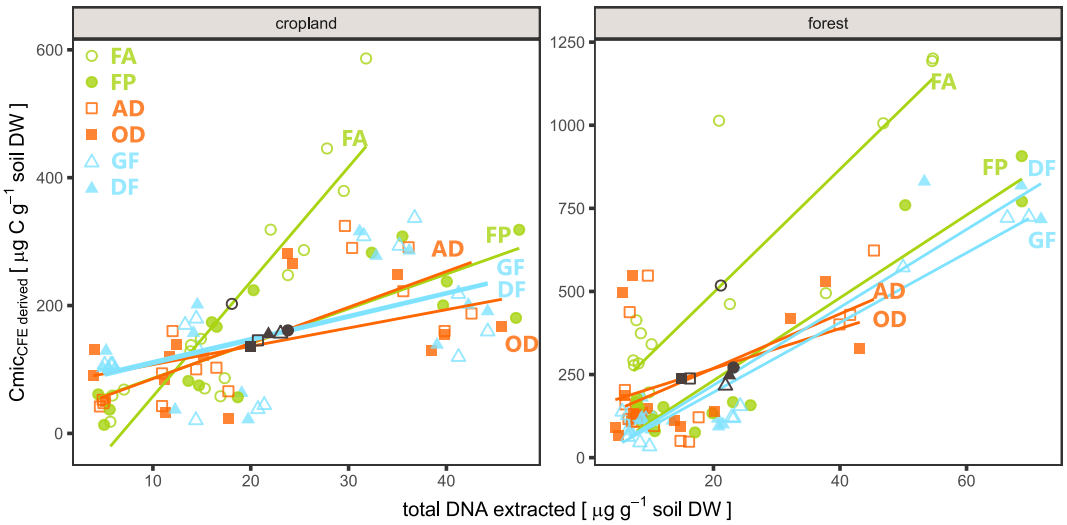

Fig. 3. Microbial biomass C (C_{mic}) derived by the chloroform-fumigation extraction method over the amount of total DNA extracted constitutes the conversion factor *fDNA*, which is used within the ^{18}O-labelling method to calculate the growth in C_{mic} over the incubation time. Coloured shapes represent single sample measurements and black shapes give group means (n = 15). Note that an individual *fDNA* was calculated for each replicate. The data are grouped by land use (facets) and pre-treatment category (colours): FA – fresh analysis, FP – fresh pre-incubated, AD – air-dry, OD – oven-dry, GF – gentle freezing, DF – direct freezing. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

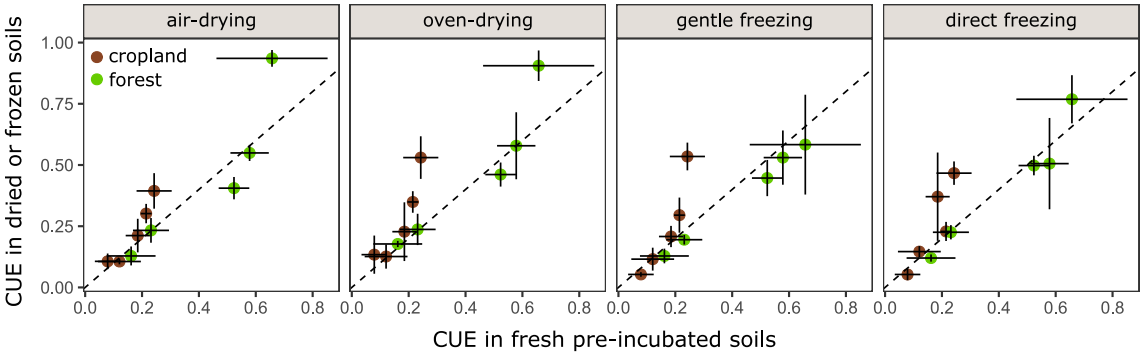

Fig. 4. Microbial CUE by ^{18}O-labelling appears to not be directly affected by frozen or dried pre-treatment. The dots give the mean CUE value of the technical replicates per site (n = 3) for the dry and frozen pre-treatments over the mean CUE value of the reference pre-treatment fresh pre-incubated. The dashed line marks the 1-to-1 line. Values above the line overestimate CUE in comparison with the fresh pre-incubated reference. Error bars indicate standard deviations.

Fig. 4 depicts that CUE values of treated samples compared with the reference scatter closely around the 1-to-1 line in both land use types. This underlines that overall CUE was only weakly affected by soil sample drying & rewetting and freezing & thawing. The *Vr* cropland site was located to the left of the 1-to-1 line in all pre-treatments. Two forest outliers were detected, which were even higher than the supposed maximum value of soil microbial CUE. These two outliers originated from the coniferous forest at the *Me* site, which is classified as Podzol. The *fDNA* values of *Me* forest fresh analysis, air-drying and oven-drying were around 44, 57 and 76 respectively, exceeding the overall range of *Me*, which was between 2 and 15 (Fig S2). For the dried *Me* forest samples, high *fDNA* values were based on high C_{mic} estimates from the CFE method. In contrast, the amount of total DNA extracted in *Me* forest did not differ with pre-treatment, i.e. no increase between fresh analysis and air-drying or oven-drying. Although the amount of total DNA did not increase with drying, a greater incorporation of ^{18}O into the DNA from air-drying and oven-drying in *Me* forest soils was identified, indicating higher microbial growth rates (Fig. 2C).

3.3. Estimated abundance of fungi, bacteria and archaea by qPCR

To reduce site variances and focus on differences between the pre-treatments, a decision was taken to consider the amount of gene copies per ng DNA rather than the amount of gene copies per g soil DW. This was acceptable because the total amount of total DNA extracted per g soil DW was not significantly affected by the different pre-treatments (Fig. S2; Table S3). Regarding the assessment of gene copy numbers, the microbial community reference served as the reference and was included in the statistical analysis.

Both drying pre-treatments significantly increased the gene copy numbers of fungi in the forest, while in cropland soils the copy numbers of fungi were not affected by drying & rewetting ($p < 0.001$) (Fig. 5A). In air-dried forest samples, the mean increase from the microbial community reference with air-drying was 77.7% ± 27.7%. In oven-dried forest samples, the gene copy numbers of fungi doubled on average (198% ± 258%) compared with the microbial community reference. The increase in the estimated abundance of fungi in forest soils after drying & rewetting was also reflected by a significantly increased F:B ratio (Fig. 5B).

Furthermore, the increase in the gene copy numbers of bacteria with pre-treatment in comparison with the microbial community reference was quantified, and was found to be more distinct in cropland soils (Fig. 5C). While the gene copy numbers of bacteria and fungi were both significantly affected by pre-treatment and land use, those of archaea only depended on land use (Fig. 5D, Table S3).

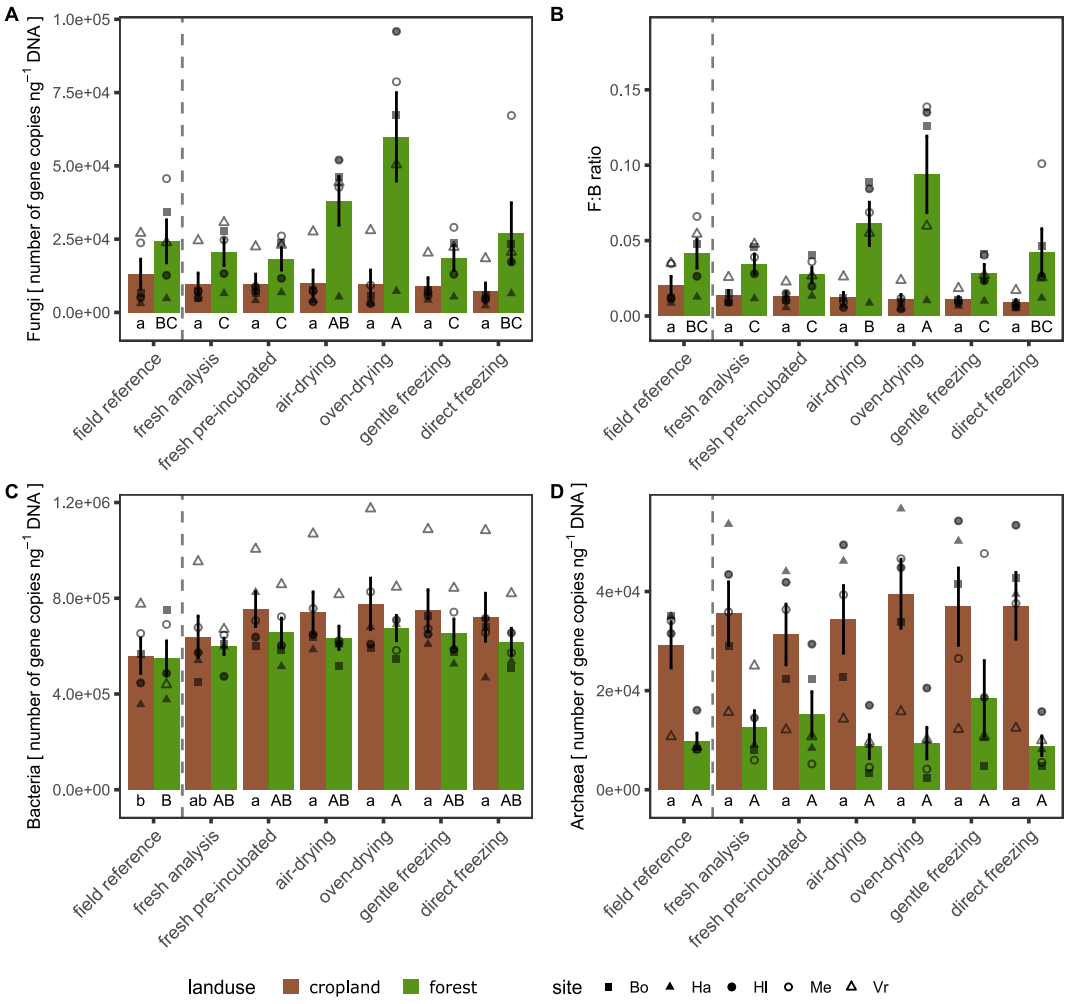

Fig. 5. Effect of soil sample pre-treatment on soil microbial community: **A)** gene copy numbers of fungi, **B)** fungi-to-bacteria ratio (F:B ratio), **C)** gene copy numbers of bacteria, and **D)** gene copy numbers of archaea for microbial community reference (field reference) and pre-treatments according to land use. Estimated abundance data were derived by qPCR and here are given as number of gene copies per ng DNA to exclude site differences. The bars indicate the overall mean and error bars give the standard error per pre-treatment (sites, n = 5) according to land use. Letters below the bars indicate significant differences according to estimated marginal means ($\alpha < 0.05$) between pre-treatments within the same land use type. Differently shaped points represent site means (replicates, n = 3). Statistical analyses were conducted on log-transformed data, except for the F:B ratio.

4. Discussion

4.1. Overall reducing effect of pre-incubation but increasing effect of drying & rewetting on CUE associated parameters

Respiration rate as well as microbial growth and overall living microbial biomass were lower in the fresh pre-incubated samples than in the fresh analysis samples. It is likely that considerable amounts of labile and easily degradable SOC were mineralised during the storage period and subsequent two-week pre-incubation, causing the observed decline in microbial respiration rate, growth and overall living biomass. It is acknowledged that the experimental design did not allow a clear distinction to be made between the effects of pre-incubation and storage as fresh analysis was directly analysed, while pre-incubated samples were analysed site-wise, resulting in different storage durations (1–5 weeks) within fresh analysis and pre-incubated samples between sites. Nonetheless, a pre-incubation effect was visible across all sites. According to Rousk and Jones (2010), K_2SO_4-extractable C can be considered as a proxy for available SOC. The observation that K_2SO_4-extractable C decreased with pre-incubation reinforced the assumption that 14 d of pre-incubation resulted in the consumption of a considerable part of the easily available C, limiting microbial metabolism after this period of pre-incubation. Pre-incubation of soil samples after disturbance is recommended to overcome short-term surges of metabolism due to disturbance and achieve a steady state again (Franzluebbers, 1999), but should be kept as short as possible to avoid resource depletion. Overall, the 14 d long pre-incubation period might have surpassed the sweet spot of this balance.

The observed increase in respiration rate after drying & rewetting is in line with numerous studies (Fierer and Schimel, 2003; Meyer et al., 2019) and is referred to as the Birch effect (Birch, 1958). This pulse of respiration has been found to last up to several days, with observations ranging from 1 to 3 d (Fierer and Schimel, 2003; Franzluebbers, 1999) up to 11 d (Meyer et al., 2019) after the rewetting event. Interestingly, in this study increased respiration rates were observed in dried & rewetted samples compared to other pre-incubated samples after the end of a 14 d recovery period from the rewetting event.

The increased respiration with drying & rewetting is accompanied by a surge in mineralisation, which is believed to be based on the increased availability of SOC and other nutrients (Birch, 1958; Powlson and Jenkinson, 1976). More recent studies have also shown air-drying to increase the amount of DOC (Kaiser et al., 2001; Miller et al., 2005). Increased K_2SO_4-extractable C concentrations with drying & rewetting in non-fumigated samples indicate the availability of supplementary C

sources after drying in otherwise depleted soils, as seen in the reduction in $C_{Respiration}$ with pre-incubation.

Mechanisms of increasing C availability after soil drying are considered to have physical or biological origins. While microbial cells killed by soil desiccation make only a minor contribution to surges in mineralisation, non-biomass SOC is a major source of C mineralised during drying & rewetting cycles (van Gestel et al., 1991; Wu and Brookes, 2005). Soil drying causes physical soil aggregate disruption and the cracking of organic colloids, releasing protected soil organic matter and making it more susceptible to microbial degradation (Powlson and Jenkinson, 1976; van Gestel et al., 1991; Balesdent et al., 2000). Therefore, the observed increase in K_2SO_4-extractable C with air-drying and oven-drying was expected to be linked to soil texture, i.e. clay content, which is an indicator of micro-aggregates (Totsche et al., 2018). However, this relationship was not supported by the statistical analysis in the present study, possibly due to the limited number of different soils ($n = 5$). Oven-drying was shown to have stronger effects than air-drying, which might be due to stronger physical stress during oven-drying at higher temperatures of 40 °C. It is to be noted, that soil sieving as a routine soil homogenisation step is an important source of physical disruption, which might potentially affect historically undisturbed forest soils stronger than cropland soils. In this study, all samples including the microbial community reference had undergone sieving. Therefore, differences observed between pre-treatments cannot be attributed to sieving.

In the present study, mass specific growth rate increased significantly with drying & rewetting, while C_{mic} was not affected by drying, except for the *Me* and *Ha* forest soils. Mass specific growth rate is based on DNA, more precisely the proportion of DNA produced within 24 h per total DNA extracted, and thus gives information about the relative microbial growth. It is a process-based parameter, while C_{mic} is potentially more stable. In soils with low microbial biomass, even a large mass specific growth rate can result in marginal absolute C_{mic} changes. Assuming all K_2SO_4-extractable C is available for microbial metabolism, it was expected that the more C was extractable, the more microbial growth could be observed. However, extractable C was only weakly correlated with mass specific growth rate or $C_{Respiration}$. This may indicate that not all C extracted from non-fumigated soil by K_2SO_4 is available and can be used for microbial metabolism.

Overall, air-drying was shown to impact the assessed parameters less than oven-drying, and is therefore preferable.

4.2. No significant effect of pre-treatment on microbial CUE

Although single parameters of microbial metabolism, such as respiration or mass specific growth rate were affected by drying & rewetting, CUE estimates did not reveal any significant general response to pre-treatment over all sites. It should be noted that the selected sites only represent a sample from a specific range of temperate arable and forest soils. It cannot be precluded, that soils from other biomes or with different properties would respond differently. While CUE is a ratio representing the relative share of C uptake for microbial growth, it can reflect microbial conditions that are fundamentally different. Same estimates can be derived from very high values of respiration and growth or very low values of the same variables. Notwithstanding, the finding that pre-treatment does not affect the sharing of C resources within the microbial metabolism is of utmost importance. It implies that CUE values, e.g. to assess soil management effects, can potentially be assessed from dried archived soil samples. However, the longest storage timespan until the start of pre-incubation in the present study was five weeks, thus potential storage time effects could not be investigated.

Nonetheless, a tendency of CUE reduction with 14 d pre-incubation was observed. In general, a decline in CUE with pre-incubation seems plausible as the depletion of labile C sources during the pre-incubation period potentially affects overall microbial metabolism. The quality of soil organic matter is believed to have a significant impact on CUE (Takriti et al., 2018) with water-soluble small molecules, i.e. sugars or amino acids, considered to be more readily metabolised by microbes, thus being considered as “labile” (Cotrufo et al., 2013). Polymeric substrates require an initial energy investment by producing exoenzymes, which reduces the energy yield available for growth for the respective organisms.

Slightly reduced CUE values with pre-incubation were attributed to altered *fDNA* values, which was more pronounced in forest soils. While C_{mic} decreased with pre-incubation, which was however only significant in forest soils, the amount of total DNA extracted generally remained unaffected by the pre-treatments, with only minor reductions in the dried forest soil samples. In contrast to Pesaro et al. (2003), who found a 24% reduction in total DNA after freezing & thawing, DNA was not reduced in frozen pre-treatments compared with fresh pre-incubated samples. It should be noted that the total DNA as measured not only originates from viable growing microbial cells, but also from metabolically inactive cells, dead cells and extracellular DNA that is stabilised by sorption to surface-active soil particles (Nielsen et al., 2007). Extracellular DNA from lysed cells has been shown to persist for 60 d or more (Romanowski et al., 1993; England et al., 2004). There may be different reasons for the stability of the DNA pool, and it does not necessarily indicate the absence of pre-treatment effects on the soil microbes. Lysed or generally killed microbial biomass by pre-treatment effects potentially fosters microbial growth of other organisms recycling substrates from this necromass, and therefore could also lead to a net zero change in DNA. This might explain the *fDNA* shifts and thus the slight, yet insignificant changes in CUE with pre-incubation.

These results underline the importance of using soil-adjusted rather than rigid *fDNA* values. In the original protocol, Spohn et al. (2016a) fitted a linear regression for the relation of C_{mic} to total DNA extracted, which was then considered for the conversion of produced DNA into C_{Growth}. They used a single function to fit data of different soils and land use types. The present results demonstrate high variability in *fDNA* values between sites, land use types and pre-incubated vs. freshly analysed samples, which is visible in the wide scattering of points (Fig. 3). Therefore, this study supports the approach of individual *fDNA* values applied in more recent studies (Poeplau et al., 2019; Zheng et al., 2019; Canarini et al., 2020). To our knowledge, this is the first study to describe a significant impact of pre-incubation on the *fDNA* conversion factor.

4.3. Pre-treatment effects on gene copy numbers of fungi, bacteria and archaea

To investigate which pre-treatment fits best if the ^{18}O-labelling approach is used both to generate parameters of microbial metabolism and for further metagenomic analyses, the effect of pre-treatment on the abundance of bacteria, archaea and fungi was evaluated based on the copy numbers of their genetic markers. We acknowledge that gene copy numbers are not equivalent to the abundance of organisms. However, changes in gene copy numbers were considered a rough proxy for changes in the microbial community. A significantly greater estimated bacterial abundance, as indicated by gene copy numbers, was observed with pre-incubation. Increases in the gene copy numbers of bacteria were already observable after one day in fresh analysis samples compared with the microbial community reference. Incubation conditions might have fostered bacterial growth. It is suspected that the sieving process affects the abundance of bacteria and increases their relative proportion at the expense of relative fungi abundance (Thomson et al., 2010; Blaud et al., 2017). Our data, however, indicate that pre-treatments affected estimated abundance beyond the sieving effect since all pre-treatments, including the microbial community reference, were sieved prior to further analyses. Interestingly, freezing & thawing, which is recommended for the analysis of soil DNA and microbial communities, did not result in values any closer to the microbial community reference treatment than those of the other pre-treatments.

In the case of the microbial community, drying & rewetting mainly increased the estimated abundance of fungi in forest soils, as indicated by qPCR. While it is generally assumed that many soil bacteria metabolise and grow with labile C sources, most soil fungi particularly utilise more chemically complex C sources (Brant et al., 2006; Paterson et al., 2008). Hicks et al. (2019) investigated whether the fungal growth response to drying & rewetting is suppressed by bacteria feeding on released labile C. They used a bacteriostatic compound (bronopol) to eliminate bacterial competition in two different soils and found a negative correlation between bacteria and fungi, suggesting that competition with bacteria can constrain the fungal growth response to drying & rewetting. One of the soils exhibited a pronounced fungal growth response to drying & rewetting even in the control treatment, i.e. without the application of bactericide, which was comparable to specific site responses in the present study. This indicates that the composition of the microbial community creates a complex web of interdependencies, which determines the effect of drying & rewetting on the abundance of individual taxa. This is supported by the significant interactive effect of pre-treatment and site on the estimated abundance of fungi in our forest data. Here, soil DNA was used only to quantify gene copy numbers of the microbial domains, i.e. bacteria, archaea, fungi. However, to elucidate the impact of pre-treatment on the soil microbial community composition, more detailed information on the responsive taxa should be gathered from subsequent metagenomic DNA sequence analyses.

4.4. Methodological limitations for the assessment of ^{18}O-CUE due to soil properties

Despite these overall clear findings, confusing discrepancies were discovered in the *Me* site data. The mean CUE values of fresh analysis, air-drying and oven-drying pre-treatment of the *Me* forest site exceeded the supposed stoichiometric maximum value of 0.88 (Gommers et al., 1988). Geyer et al. (2019) report CUE estimates derived from the ^{18}O-labelling method to be generally lower (<0.4) than from substrate-specific approaches such as ^{13}C-labelling. Thus, *Me* forest CUE estimates derived by ^{18}O-labelling > 0.88 appeared even more unrealistic. *Me* forest *fDNA* values were around 70 in dried pre-treatments, while *fDNA* values as described in the literature range between 2.2 and 16.33 (Joergensen and Emmerling, 2006; Spohn et al., 2016a, 2016b; Chen et al., 2020). As *Me* forest *fDNA* values exceeded the assumed range and low total DNA values do not fit with high C_{mic} data, it is assumed that analytical errors were made either within DNA quantification or CFE.

Me forest shows all the typical soil characteristics of what is known as black sand (Jacobs et al., 2018). Black sands are defined by a C:N ratio ≥12 and a sand content ≥70% and are classified as Podzol or Plaggic Anthrosols according to the *WRB* classification (IUSS Working Group WRB, 2015). These soils have been reported to depict low microbial biomass C (derived from field-fresh soil) at high SOC contents, as major proportions of the organic material in these soils do not support a microbial population (Springob and Kirchmann, 2002). The organic matter in these soils often originates from heath or peat and there are indications that standard protocols of soil analyses do not work. For example, DNA extraction in this study also yielded brownish eluate for this site, indicating contamination by humic substances, which could potentially have hindered successful DNA quantification (Bachoon et al., 2001). However, the DNA was purified from humic acids and the DNA concentration of these *Me* samples was not extraordinarily high, which would have been a typical indicator supporting this hypothesis rather than the observed low values. Another explanation could be a potential error in separation of horizons, with some of the litter layer potentially being incorporated into the bulk soil samples in forest plots. Leckie et al. (2004) report that C_{mic} was not correlated with total DNA in forest humus. The authors assumed this lack of correlation was caused by the high abundance of fungi in the forest humus, as DNA concentration per unit biomass is much more variable for fungi than for bacteria. With regard to the *Me* data, this hypothesis was also supported by the increased abundance of fungi in air-drying and oven-drying pre-treatments. Another indicator for a potential bias in the CFE method is, that the *Me* forest soil was the only soil where C_{mic} increased with drying.

The methodological limitations of DNA extraction and CFE-derived microbial biomass C need especial consideration in the novel method of ^{18}O-CUE, where a large number of single parameters has to be assessed and combined in the end.

The finding of a significant pre-incubation effect on *fDNA* estimates demonstrates the high variability in the *fDNA* value. Although, it is believed that the high *fDNA* values above the range cited in the literature are plausible (especially for fungi-marked communities), high *fDNA* values in *Me* forest soils are questionable. Further investigations of the microbial community composition may reveal whether high *fDNA* values are linked to specific fungal groups potentially only abundant in these high *fDNA* treatments, such as the dried *Me* forest soil. CUE estimates above the theoretical maximum indicate a potential limitation of using *fDNA* values derived from CFE, as microbial biomass reacts much more slowly to environmental changes than growth. This is in agreement with Pold et al. (2020) who question the assumption that *fDNA* is representative of the community that grew during incubation with $^{18}O–H_2O$. As we could show, the proportion of microbial biomass C per unit DNA is far from being fixed and stable. As the CUE refers to the total of C directed to growth, not only the total of C incorporated into microbial DNA, DNA alone does not serve as replacement for C_{mic} values in CUE assessment.

4.5. Are forest and cropland soils similarly affected?

As a large number of studies focus on treatment comparisons, the aim here was to establish whether pre-treatment biases on parameters derived by the ^{18}O-labelling method are proportional between treatments, and whether forest and cropland respond equally to pre-treatment. Regarding the effect of pre-treatment on parameters of microbial metabolism ($C_{Respiration}$, C_{mic}, CUE, C_{Growth}), no indication was found of interactive effects of land use and pre-treatment. Consequently, cropland and forest soils responded similarly to pre-treatment. This is in line with Meyer et al. (2019), who tested the effect of sieving and storage conditions on soil respiration and its Q10 at 14 sites – five cropland sites, four grassland sites and five forest sites – representing a range of soil textures and SOC contents. In their study, all soils were affected similarly, regardless of land use type.

The present results indicate that data about microbial metabolism can be derived from fresh, dried & rewetted, and frozen & thawed soil samples for the purpose of comparative studies. For topsoils, a change in land use type *per se* is among the most extreme treatments that can be investigated, since it affects almost all soil properties, including microbial communities (Szoboszlay et al., 2017). Additionally, redistribution of organic C compounds with routine laboratory homogenisation procedures would likely be greater in forest soils than traditionally disturbed agricultural soils. However, our study confirmed that the relative change due to pre-treatment was fairly similar in both land use types. It is therefore assumed that the result obtained would also be valid for other, less extreme *in-situ* treatments, such as changes in agricultural management.

However, the linear mixed-effects model approach revealed a significant interactive effect of land use and pre-treatment on the gene copy numbers of fungi, resulting in an elevated estimated abundance with drying & rewetting in forest soils only, while cropland soils did not exhibit such increases. This change in microbial community composition might explain why forest soils tended to be slightly, though not significantly, more responsive to sample pre-treatment than cropland soils. Fungal dominated soils, such as the forest soils in this study, might be more affected by pre-treatment.

5. Conclusions

Although parameters such as microbial biomass and respiration were biased by pre-treatment, overall the CUE estimates as a ratio parameter were found to be stable against pre-treatment variation. The most pronounced effect on all parameters was caused by 14 d pre-incubation, which was found to be too long to properly represent *in-situ* conditions at the time of sampling. However, overall both land use types were affected in a similar way, so that relative differences between treatments should be widely unaffected by sample pre-treatment. It is therefore concluded that, CUE estimates can be derived from soil samples regardless of whether they are dried & rewetted, frozen & thawed or stored fresh. This opens up the opportunity to choose time-saving, convenient and less expensive handling of samples without risking major biases between treatments. Nonetheless, one restriction of these results is that there was a different effect of pre-treatment on the estimated abundance of fungi between forest and cropland soils. Analyses of the soil DNA derived from the ^{18}O-labelling method by qPCR allowed information to be gathered on the abundance of the major taxonomic groups (bacteria, archaea, fungi) for cropland soils, while forest soils responded in a more complex way to the pre-treatments. The microbial community structure of forest soils presumably responds more sensitively to drying & rewetting, which appears to be related to the greater abundance of fungi. The data for the *Me* site may point to methodological shortcomings in ^{18}O-CUE assessment. We would like to encourage scientific colleagues to retain such outliers in published data in order to identify and overcome methodological issues.

Declaration of competing interest

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

Acknowledgments

We would like to thank the technical staff of the Thünen Institute involved in this study: Sabine Wathsack and Kerstin Gilke for processing CFE and gas samples respectively, and Jana Usarek, Karin Trescher and Britta Müller for extracting DNA and conducting PicoGreen screening and qPCR. We also appreciate the work of Dr Márton Szoboszlay, who checked for qPCR quality. Special thanks to Dr Jens Dyckmans and his colleagues at the Centre for Stable Isotope Research and Analysis at the University of Göttingen. This study was conducted by, among others, Lisa Kammann as part of her master's thesis. This work is part of the "Breaking the Ice" project funded by the German Research Foundation [grant number 401106790].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108321.

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