

Who is who in necromass formation and stabilization in soil? The role of fungi and bacteria as complementary players of biogeochemical functioning

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Abstract

Multiple global change drivers have caused a large carbon (C) debt in our soils. To remedy this debt, understanding the role of microorganisms in soil C cycling is crucial to tackle the C soil loss. Microbial carbon use efficiency (CUE) is a parameter that captures the formation of microbially-derived soil organic matter (SOM). While it is known that biotic and abiotic drivers influence CUE, it remains unclear whether bacteria, fungi and their interactions influence the formation of microbially-derived SOC and its persistence in soils. Here, we combined the inoculation of distinct communities (a biotic factor) grown at different moisture levels (an abiotic factor) to manipulate the formation of microbial necromass in a model soil. In a follow-up experiment, we then evaluated the persistence of this previously formed microbially-derived C to decomposition. While we show that necromass formation reflects the microbial community composition, the SOC formed within the most complex community of bacteria and fungi seems to be more resistant to decomposition compared to the SOC formed within the simpler communities (bacteria and fungi simple community, bacteria only and fungi only communities). Moreover, fungal necromass proved to be more thermally-stable than bacterial necromass, if this necromass is formed with both bacteria and fungi present. Our findings reveal that although abiotic factors can influence microbial physiology, the biological origin of microbially-derived C and the co-occurrence of fungal and bacterial growth were the stronger drivers explaining SOM persistence in these soils, suggesting the importance of microbial succession in SOC stabilization.

Keywords: global change; microbial carbon use efficiency; necromass; carbon cycling; climate change; ecosystem resilience; soil organic matter

Introduction

Soil organic matter (SOM) accounts for the largest terrestrial C stock [1–3]. However, due to a combination of land conversion [4], intensification of agricultural practices [5] and global warming [6, 7] SOM stocks are declining globally. In order to evaluate the actions needed to reverse this trend, it is necessary to understand the processes by which SOM is formed and stabilized.

SOM formation and stabilization occur through microbial processing [8], making the formation and turnover of microbial

biomass (i.e. necromass) a key driver of soil C dynamics [9, 10]. Indeed, up to 50% of the more stable SOM pools consist of microbial necromass [10, 11], thus the rate and efficiency with which soil microorganisms incorporate plant inputs into their biomass rather than releasing it as CO₂ thus play a role in regulating the input rate to this key SOM pool [8, 12–15]. More diverse microbial communities allocate more C to growth in relation to respiration than species-poor communities [16], which could indicate higher rates of SOM formation under more complex communities. Furthermore, the fate of even simple C input

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compounds is shaped by the microbial community present, with distinct substrate conversion efficiencies and SOM chemical composition resulting under different microbial communities [15–17]. This has in part been attributed to fundamental differences in the resource requirements and metabolisms of bacteria versus fungi [18–20], but variation within bacterial communities as well as interactions between these two main groups are also likely to play a role [17]. For instance, fungi may produce compounds that bacteria then “cross-feed” on, increasing the efficiency of bacteria’s growth [21]. If microorganisms mobilize biomass building blocks from microbial necromass within the SOM [22], cross-feeding might be also a mechanism enhancing SOM turnover. While previous results suggest that decomposition of fungal residues is a regulator for soil functioning and C accumulation in soils [23], it becomes crucial to evaluate the role of fungal and bacterial interactions in this process.

Abiotic factors are known to directly influence microbial metabolism and by modifying the environment, they can also influence the biotic interactions [24]. For example, a study showed that drought can modulate the relationship between bacterial diversity and CUE, with CUE being positively correlated with bacterial diversity under high moisture but not under drought [16]. Drought is also known to have different effects on soil fungal and bacterial communities [25] and their growth efficiency [26, 27]. Soil bacterial communities are expected to be more affected by drought as they are more susceptible to substrate limitation [28], desiccation and/or water deficit compared to fungal growth which is less dependent on the water film due to hyphal growth [29]. Abiotic factors such as wet/dry cycles will also impact soil aggregate structure and formation [30]. For example, fungal hyphae was showed to be able to enmesh soil particles even under drier conditions [31]. Soil aggregation is an important parameter contributing to the persistence of SOM in soils [32].

The study of the formation of more stable components of SOM and their turnover, despite challenging [33], is a growing research area [34]. More recently, SOM ramped thermal analysis have been suggested as a way to capture carbon pools differing in their residence-time and “biologically-reactivity” [35], as it provides a metric of SOM intrinsic thermal stability [35–37]. To gain further insights into the roles of fungal and bacterial communities for C cycling through soils we designed an experiment where we manipulated microbial communities and assessed its impact on CUE, necromass, and aggregate formation as well as the thermal stability of the formed SOM. The experiment consisted of two phases: in phase I, we inoculated a sterile C-free soil with four distinct communities (complex community of fungi and bacteria, simple community of fungal and bacterial strains, bacteria only, and fungi only communities). For phase II, we then X-ray sterilized the soils, re-inoculated them, and evaluated how the new microbial community degraded the microbially-derived SOC formed during the phase I (Fig. 1). We hypothesized that: (i) Fungi-dominated community produce a higher content of necromass compared to bacteria-dominated community; (ii) more complex microbial communities result in a microbial-derived SOC that is more persistent in the soil due to enhanced SOC turnover; and (iii) Co-occurrence of fungal and bacterial growth enhances potential SOC stabilization due to enhanced necromass-mineral interactions [38]. We used SOM thermal-stability as a proxy for SOM stabilization and microbial-driven aggregate formation as a proxy for necromass-mineral interactions.

Materials and methods

Model soil, inoculation, and incubation conditions

We created a microbe and C-free soil to evaluate how distinct microbial communities generate distinct microbial-derived-SOC. 90% acid-washed sand (0.1–0.8 mm) was mixed with 10% bentonite clay (montmorillonite) treated with calcium chloride and autoclaved three times with a minimum interval of 48 h between cycles.

We created four different microbial community treatments. First, a simple community of bacteria (“B_{simple}”) was created consisting of the mixture of two bacterial strains *Streptomyces* sp. (gram-positive Actinomycetota DSM 687) and *Microvirgula aerodenitrificans* (gram-negative Pseudomonadota DSM 736). The genus *Streptomyces* is commonly found in soils and decomposing litter and belongs to the phylum Actinomycetota. *Streptomyces* exhibit high metabolic diversity, growth involving the formation of multicellular filamentous hyphae and the ability to form spores. *M. aerodenitrificans* belongs to the Pseudomonadota phylum and has an atypical respiratory type of metabolism in which oxygen and nitrogen oxides can be used simultaneously as terminal electron acceptors. Then, a simple fungal (“F_{simple}”) inocula treatment was generated using the fungal strain *Trichoderma koningii* (DSM 63059). *Trichoderma koningii* is a soil saprotroph commonly known as a fast-growing and effective biocontrol agent. The simple bacterial and fungal community (“BF_{simple}”) were generated by combining inocula half of the volume of inocula used in the B_{simple} and F_{simple} communities. We also created a complex community of fungi and bacteria (“BF_{complex}”) by shaking 2 g soil derived from the TwinWin experiment [39] in 50 ml of sterile deionized water (Supplementary Table 1). We aimed to inoculate the model soils with distinct communities at the same abundance per gram of soil. To achieve this, we determined the corresponding colony-forming units (CFUs) at two different optical densities during the exponential growth phase of bacteria and fungi in liquid media, in order to calculate the necessary inoculum volumes to achieve an abundance of $\sim 1.0 \times 10^7$ CFUs per gram of soil (Supplementary Fig. 1). The bacterial and fungal strains were purchased at the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and were grown in liquid media following DSMZ guidelines. For the BF_{complex} community we performed serial dilutions with a soil inocula and performed CFU counting to determine the ratio of inocula:soil needed to reach $\sim 1.0 \times 10^7$ CFU g⁻¹ soil. Despite our efforts to achieve similar inoculate densities at the beginning of the experiment, we might underestimate the CFU of the natural soil inocula compared to the other inocula as some species might not grow on the plate media but might growth on the model soil. Our experimental design and measurements do not allow us to fully exclude this possibility. Nevertheless, previous inoculation experiments have shown that inocula with distinct densities reach a same abundance when substrate levels is similar [16]. We also had non-inoculated microcosms which did not receive an inoculum (uninoculated controls; 8 samples) which we used to verify that these soils were sterile in the beginning of the experiment by evaluating CO₂ production in these microcosms. Microcosms were incubated at two different water content treatments (40 and 80% WHC) in a full factorial design with four replicates per treatment (4 community treatments \times 2 moisture levels \times 4 replicates = 32 biological samples) (Fig. 1). To allow for maximum utilization of nutrients, only one substrate addition was performed at the moment of inoculation.

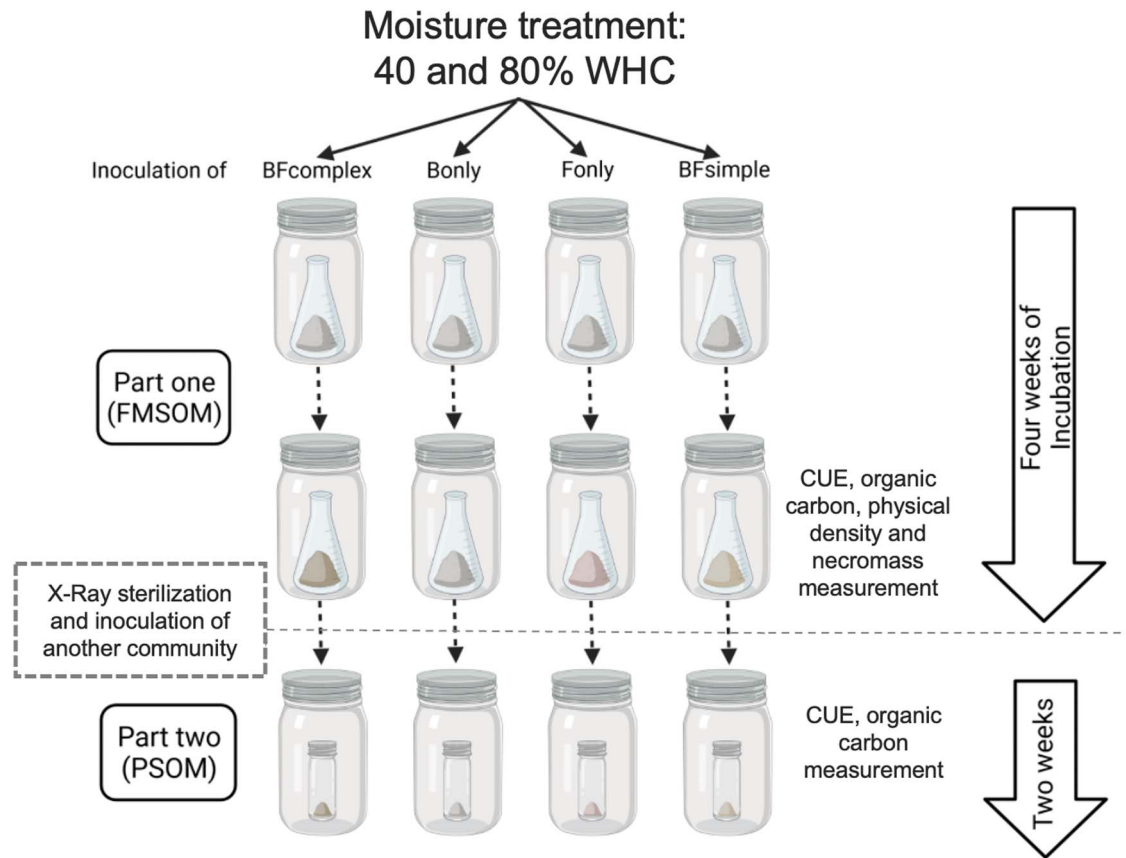


Figure 1. Experimental design. Distinct communities with equivalent densities were inoculated in an initially microbe-free and C-free soil. The inoculum was manipulated by extracting a complex bacteria and fungal community from a natural soil (“BF_{complex}”); inoculating two bacterial strains (“B_{only}”) (representing an Actinomycetota and Pseudomonadota: *Streptomyces* sp and *Microvirgula aerodenitrificans*, respectively); inoculating one fungal strain (“F_{only}”) (representing Ascomycota: *Trichoderma koningii*); combining the B_{only} and F_{only} inoculums into a “BF_{simple}” inocula. These communities were incubated during four weeks in a phase referred as “FMSOM”. We measured CUE, quantified the C remaining and its thermal signature as well as the necromass formed during the incubation and the soil aggregate formation (via physical fractionation). At the end of FMSOM all communities were X-ray sterilized. A new community derived from a natural soil was inoculated to estimate the persistence of the microbially-derived carbon formed during the first weeks of incubation (persistence of soil organic matter; PSOM) and we performed this by measuring the respiration, growth and CUE at this second incubation phase.

Each 10 g model soil microcosm was amended with Tryptic soy broth medium and glucose to result in 4.8 mg C g⁻¹ soil.

Experiment phase I: formation of microbially-derived SOM

We used an array of complementary methods to characterize the physiology of microbial communities, and the quantity and quality of microbially-derived SOM. Below, we describe in more detail the distinct methods. This first phase of the experiment is denominated “Formation of Microbially-derived SOM” (FMSOM). Microcosms were incubated for four weeks and microbial activity monitored by CO₂ flux measurements. At the end of the FMSOM incubation phase we determined the CUE of the communities with the ¹⁸O-H₂O method [40]. We evaluated the quantity and quality of SOC with the ramped thermal rock-aval pyrolysis method [36], measured the amino sugars produced by the distinct microbial communities following established methods [41], and estimated the aggregate formation by density fractionation [42].

Cumulative respiration

We evaluated microbial activity during the two phases via cumulative CO₂-C production. Microcosms were placed inside a jar (0.75 l) containing a septum port. The respiration from these jars was measured by sampling 20 ml gas samples every 48 h, with

the headspace flushed to prevent excessive accumulation of CO₂ after every measurement. Jars without any microcosms were also closed in order to measure the starting CO₂ concentration. The CO₂ concentration inside the jars was measured by gas chromatography (Agilent Technologies 7890A).

Growth, respiration, and CUE measurements

We used the substrate-independent method H₂¹⁸O method to evaluate the community CUE [40]. The day after microcosm harvest, two 0.3 g soil aliquots were weighed into 15 ml falcon tubes and placed in a large tube. Soils were brought to 40% or 80% WHC and 20% H₂¹⁸O with a combination of milliQ water and H₂¹⁸O. Control soil samples received milliQ water. After adding water, the tubes were sealed and incubated at room temperature for 48 hr. After incubation, 25 ml of the headspace was sampled for respiration. The soil samples were then frozen at -80°C until DNA extraction. CUE measurements using the H₂¹⁸O-CUE method estimate the new microbial biomass produced during the incubation period based on ¹⁸O-DNA. DNA was extracted from all soils incubated with ¹⁸O-water (i.e. technical duplicates) and soils incubated with ¹⁶O-water using the Qiagen Power Soil kit and technical duplicates were pooled before quantification using Qubit. DNA δ¹⁸O values were measured using a Flash IRMS elemental analyzer operated in pyrolysis mode coupled to a Delta

V Plus isotope ratio mass spectrometer (Thermo Fisher, Waltham, MA, 410 USA) at the Stable Isotope Ecology Laboratory, University of Basel, Switzerland, and results are reported relative to Vienna Standard Mean Ocean Water (VSMOW) in ‰. Long term instrumental precision for the laboratory for non-¹⁸O-enriched analyses is 0.2‰. Analytical precision for ¹⁸O-enriched samples measured as a part of this study was 1.57‰ (n = 6). CUE was calculated using a conversion factor microbial biomass carbon:DNA ratio of 10.9 according to Spohn et al., 2016 [40].

SOM quantity and quality

We used ramped-thermal rock-avalanche pyrolysis to evaluate SOM quality and quantity [36, 37] at the end of the first incubation phase, FMSOM, and the second incubation phase, in which we evaluated the persistence of microbially-derived SOM (PSOM). Soils were dried at 65°C and crushed to a fine powder in a mortar and pestle. Between 50–70 mg soil was pyrolyzed over a temperature ramp from 200 to 650°C, followed by combustion to 850°C using a rock-avalanche 6 pyrolyzer (Vinci technologies) at the Institute of Earth Sciences of the University of Lausanne (Switzerland). Hydrocarbons released during the ramped pyrolysis process were measured by a flame ionization detector (FID). We used rock-avalanche pyrolysis to calculate the total organic carbon (TOC) at the end of FMSOM and PSOM phases. In addition to TOC, we used two approaches to infer whether the amount of residual media differed between treatments at the end of Phase I. First we estimated remaining DOC by subtracting the measured cumulative respiration, necromass and microbial biomass formed during the incubation [41]. Second, we measured absorbance at 254 nm as a proxy of remaining dissolved organic carbon. For this, 2 g of soil was mixed with 8 ml deionized water and vortexed for 10 s. The supernatant was removed, filtered through a 0.22 μm PES filter (Merk Millipore Ltd., Ireland), placed in a quartz cuvette and read at 254 nm in a SpectraMax M2 plate reader using the deionized water as blank.

Microbial necromass (amino sugars) produced during incubation

Microbial necromass was extracted from freeze-dried soil (1 g aliquot) in duplicates [43]. To do this, soils were hydrolyzed (6 M HCl, 105°C, 8 h). The precipitates were removed and hydrolysates adjusted to a pH of 6.6–6.8. Then, amino sugars were dissolved in methanol and separated from salts by centrifugation. After the addition of a quantitative standard (methyl-glucamine), amino sugars were transformed into aldonitrile derivatives [44]. The derivatives were further acetylated with acetic anhydride. The amino sugars were quantified on a Trace GC 1310 gas chromatograph coupled to an ISQ mass spectrometer (Thermo Fisher Scientific, USA) using a DB-5MS column (30 m × 0.25 mm i.d., film thickness, 0.25 μm) for separation. We used myo-inositol as a recovery standard. The myo-inositol was added after hydrolysis, before derivatization. It is added at this point in the protocol to avoid the standard being sorbed to minerals or degraded during hydrolysis. The recovery of amino sugars was in the range of 95%. We evaluated the production of glucosamine, mannosamine, galactosamine and muramic acid. Galactosamine and muramic acid are expected to occur predominantly in bacteria while glucosamine is predominantly produced by fungal cells. We calculated the amino-sugar-C equivalent expected to be of fungal or bacterial origin based on Joergensen [45] considering that amino-sugar of bacterial origin corresponds to muramic acid (MurN) and a proportion of glucosamine (GlcN). Fungal GlcN was calculated by subtracting bacterial GlcN from total GlcN as an index for fungal residues, assuming that MurN and GlcN occur at a 1–2

molar ratio in bacteria [45] according to the formula while fungal amino-sugars are estimated on the GlcN according to the following formula: $\text{fungal GlcN} = \frac{\text{GlcN}}{179.17} - \frac{2 \times \text{MurN}}{245.23} \times 253.23$ where 179.17 and 253.23 correspond to the molecular weight of GlcN and MurN, respectively. We also calculated the amino sugar accumulation efficiency (AAE) using the following formula: $\text{AAE} = (\text{AminoSugar-C} / (\text{AminoSugar-C} + \text{cumulative}(\text{CO}_2\text{-C})) \times 100$. AAE is reported in percent (%).

Density fractionation

We used density fractionation approach to assess if microbial community activity promotes organo-mineral aggregation after the FMSOM phase. To distinguish the organo-mineral aggregates from the initial mineral and organic particles, we isolated meso-density fraction (1.8–2.4 g cm⁻³) because the particle density of sand and bentonite clay was >2.4 g cm⁻³ (a pilot test showed 0.47% of the initial mineral mass had the particle density <2.4 g/cc) and that of any organic compounds are <1.8 g cm⁻³ [42]. Sodium polytungstate (SPT) was used to adjust solution density (SPT-0 grade, TC-Tungsten Compounds, Germany). Following a previous protocol [42], freeze-dried samples (5 g) were shaken for 30 min with SPT solution adjusted to 1.8 g cm⁻³ to disrupt the less-stable aggregates and isolated organic matter not associated with minerals as low-density fraction (<1.8 g cm⁻³) by recovering floated materials after centrifugation (2330 g). To obtain the meso-density fraction, the remaining liquid was replaced with 2.4 g cm³ SPT solution, shaken and centrifuged following by the recovery of the floatable as previously done. The remaining material was recovered as high-density fraction. The recovered low-density fraction was rinsed with deionized water using a vacuum filtration system with 0.45 μm membrane filter. After recovering meso- and high-density fractions, the material was transferred to 250 ml bottles and mixed with deionized water and centrifuged (17,000 g) to remove the salt until the salt concentration in the supernatant was below 50 μS l⁻¹. The fully-rinsed fractions were freeze-dried for chemical analysis. For the three replicates from the four treatments (BF_{complex} dry, wet, B_{only} moist, F_{only} moist) fractionated, the mass recovery was 94.1% ± 1.0% (mean ± SD).

Experiment phase II: Persistence of microbially-derived SOM (PSOM)

After the first phase of the experiment which we evaluated how distinct communities promoted the FMSOM, we sterilized the soil with X-ray radiation (40 kGy). We decided to sterilize with X-ray to reduce structural modification of recently formed SOM if compared to more traditional methods such as autoclaving the soil [46]. These microcosms were then inoculated with a complex inoculum similar to our BF_{complex} treatment obtained from the same agricultural experimental site (Fig. 1). The soils were incubated for 15 days to evaluate how the new inoculum would consume the recently formed microbially-derived SOM. To achieve this, we measured cumulative respiration during the incubation and at the end of this period we measured the community CUE the quantity and quality of the SOM with methods described previously.

Statistical analysis

All figures and statistical models were performed in R (Version 2023.09.0 + 463) using the packages *vegan* [47], *agricolae* [48], and *ggplot2* [49]. The response variables were log-transformed to fit model assumptions. Amino sugars data were normalized by dividing by the remaining total organic carbon in a sample and are expressed as amino-sugar-C per g SOM. The effects of inoculum

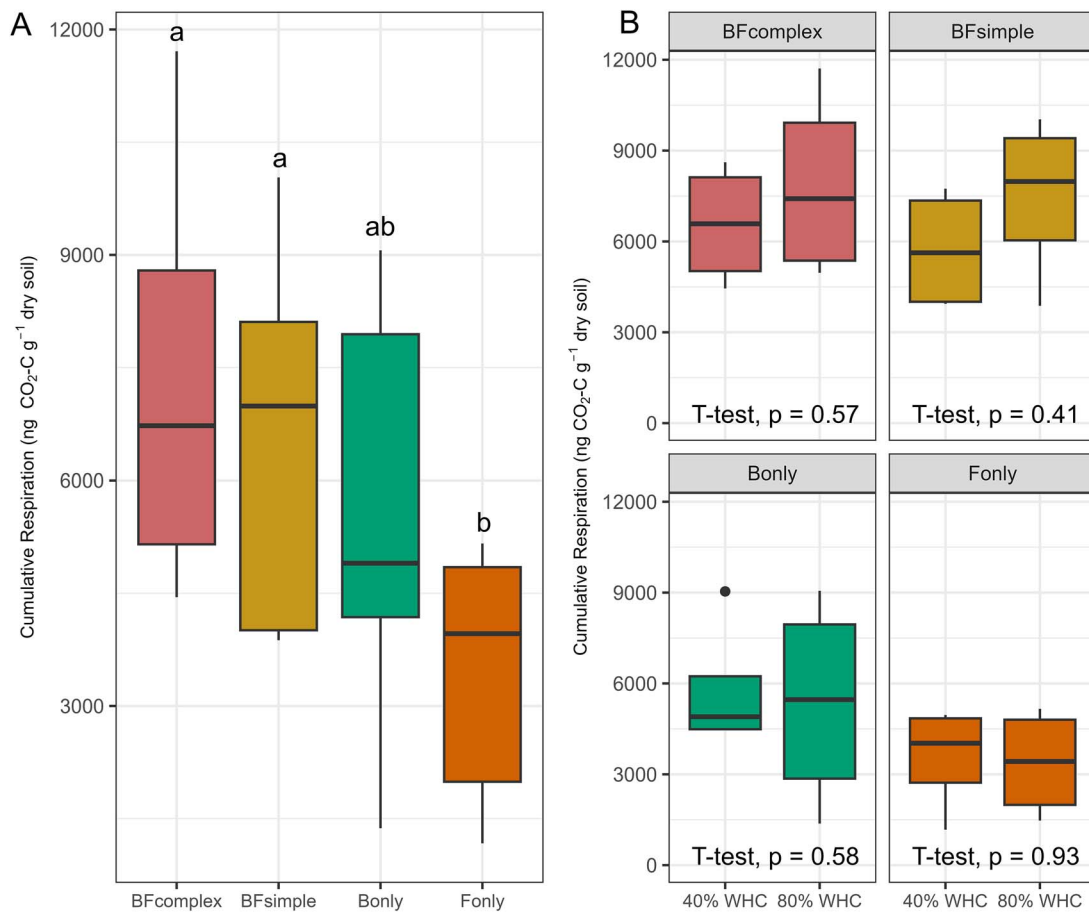


Figure 2. Respiration rates as a proxy for microbial activity. Cumulative respiration measured during first incubation phase (FMSOC) at the distinct communities (log transformed) (A). Cumulative respiration at each community type and moisture treatment (B). Significant differences between treatments are indicated with different letters (Anova followed by Tukey HSD test, $P < .05$) or significant t-test results ($P < .05$).

and moisture treatments were tested by analysis of variance (ANOVA) and *post hoc* Tukey HSD tests. Linear models were used to assess the relationship between the amino-sugar-C produced during the first phase of the experiment and the microbial physiology measurements of the second part of the experiment. To evaluate how the distinct amino sugar residues in the soil relates to the SOC thermal signature measured during the pyrolysis phase of Rock-eval we performed spearman correlations between the distinct measured amino-sugars and the FID signal captured at each temperature by ramped Rock-eval analysis. For the variation partitioning analysis, significant explanatory variables for respiration, growth and CUE were chosen by linear regression, model selection (backward) and by minimizing the Akaike Information Criterion. The statistical significance was assessed by 1000 permutations of the reduced model. The variance partitioning approach was then used to evaluate the relative contribution of those variables to explain the variation in respiration, growth, and CUE measured in the PSOM phase using the function *varpart* [50].

Results

Microbial activity and formation of microbial-derived residues during FMSOC

To assess the activity of the microbial communities we quantified cumulative respiration during the 30 days of incubation (Fig. 2A, B). Cumulative respiration did not differ among most

communities, except for the F_{only} in which respiration was lower compared to $BF_{complex}$ and BF_{simple} ($F_{3,27} = 4.770$, $P = .007$). Cumulative respiration was unaffected by the moisture treatments ($F_{1,27} = 0.194$, $P = .663$). The compilation of respiration measurements during the 30 days of incubation suggests no major difference in microbial activity across treatments except for the F_{only} treatment (Fig. 2 and Supplementary Fig. 4).

Microbial necromass was measured to assess the influence of different microbial communities on SOM formation, using amino sugars formed after FMSOM as a proxy (Fig. 3). The amino sugar signature reflected the microbial composition ($F_{3,24} = 3.778$, $P = .022$). Moisture levels and the interaction of the community with moisture showed no significant effect ($F_{1,24} = 1.728$, $P = .201$, and $F_{3,24} = 0.784$, $P = .514$). Predominantly produced by fungi, glucosamine values were higher in the fungi-dominated soils (F_{only}) than in the bacteria-dominated communities (B_{only}) (Fig. 3A–F), while galactosamine and muramic acid, which are mainly produced by bacteria, were below detection limit in the fungi-dominated microcosms (Fig. 3C–E). We calculated the amino-sugars-C expected to be of fungal or bacterial origin based on Joergensen et al. [45] (see Methods). While the production of specific amino-sugars differs between communities (Fig. 3A–D), the total amino-sugar production was not different among communities or moisture treatments (Fig. 3F). The Amino-sugar Accumulation Efficiency (AAE), defined as the total amino-sugar formed in the soils relative to the total respiration, ranged

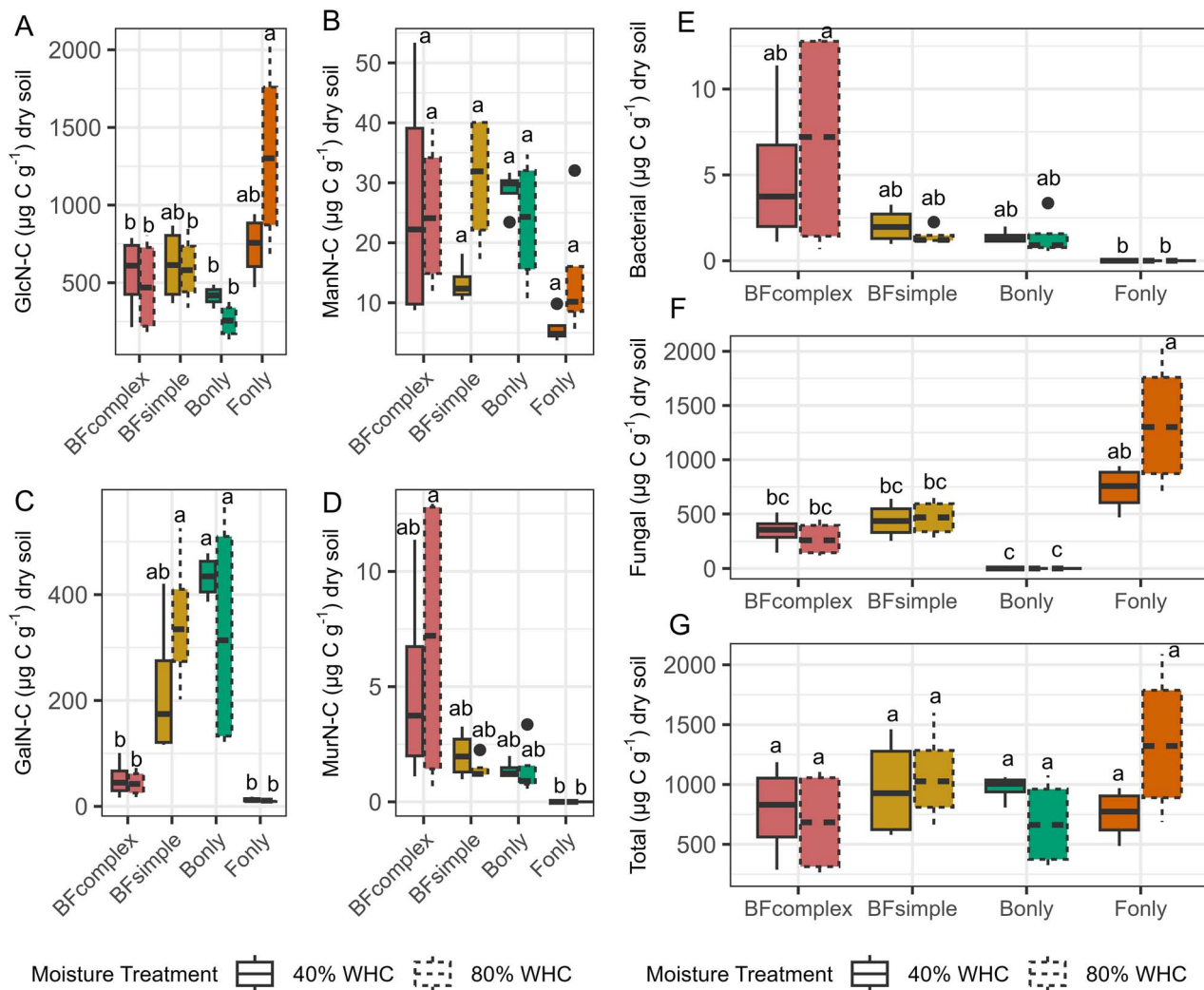


Figure 3. Amino sugar accumulated during FMSOM by each microbial community at two moisture treatments. Boxplots showing the median, the interquartile range, the minimum and maximum values. Letters indicate significant differences between communities and moisture treatment. Glucosamine (A), mannosamine (B), galactosamine (C), and muramic acid (D) produced during 30 days of incubation. Amino sugar of bacterial origin (E), fungal origin (F), and total amino sugar (G). Significant differences between treatments are indicated with different letters (Anova followed by Tukey HSD test, $P < .05$).

from 0.60% ($CI_{95\%} = [0.44-0.75]$) in the BF_{complex} up to 2.66% ($CI_{95\%} = [0.88-4.44]$) in the F_{only} treatment on average ($F_{3,28} = 7.813$, $P < .05$) (Fig. 4).

We measured the growth with the $H_2^{18}O$ -method and respiration in a subsample of soil. While we observed some differences among communities for respiration and growth no significant difference was observed within a community for the distinct moisture levels (Fig. 5A, B). Respiration rates ranged from 795 $\text{ng CO}_2\text{-C g}^{-1}$ soil h^{-1} ($CI_{95\%} = [180-1411]$) for BF_{complex} to 8140 $\text{ng CO}_2\text{-C g}^{-1}$ soil h^{-1} for B_{only} ($CI_{95\%} = [3296-12984]$) while growth rates ranged from 17 ng C g^{-1} soil h^{-1} ($CI_{95\%} = [11-22]$) for BF_{simple} to 104 ng C g^{-1} soil h^{-1} ($CI_{95\%} = [39-169]$) for B_{only}. Soil moisture did not affect respiration or growth significantly within a community. However, respiration and growth showed the opposite tendency. While respiration rates tended to be higher under the low moisture compared to high moisture treatment, growth was on average higher in moist soils compared to the dry soils (for growth: $t = 2.80$, $df = 19.50$, $P = .01$). The exception was the F_{only} treatment which showed the same pattern for both respiration and growth measurements. CUE is a compilation of growth and respiration measurements, as a consequence of these

distinct responses we observed a significantly lower CUE in the dry BF_{complex} soils compared to the wet soils (Fig. 5C). This same trend was observed for the CUE of BF_{simple} and B_{only} but the differences were not significant (Fig. 5C). F_{only} showed similar median values for CUE in both moisture treatments (average BF_{simple} = 2.0%, average BF_{complex} = 12.8%, $CI_{95\%}$ BF_{simple} = [1.0-2.9], $CI_{95\%}$ BF_{complex} = [5.2-20.4]).

Microorganisms' influence on SOM formation and its thermal stability

Total organic carbon (TOC) did not differ among treatments at the end of the first incubation phase (Supplementary Fig. 2A) suggesting that the microbial communities consumed the substrate added to the same levels during the FMSOM phase. We used the signal captured at different temperatures during pyrolysis of SOM to evaluate how it relates to the amino sugars produced during the incubation (Fig. 6). Overall, we observed that the content of amino sugars in the soil was negatively related to the signal captured at lower temperatures ($< 400^\circ\text{C}$) and positively related to the signal captured at higher temperatures ($> 400^\circ\text{C}$) with the exception of muramic acid. Further, we observed that fungal

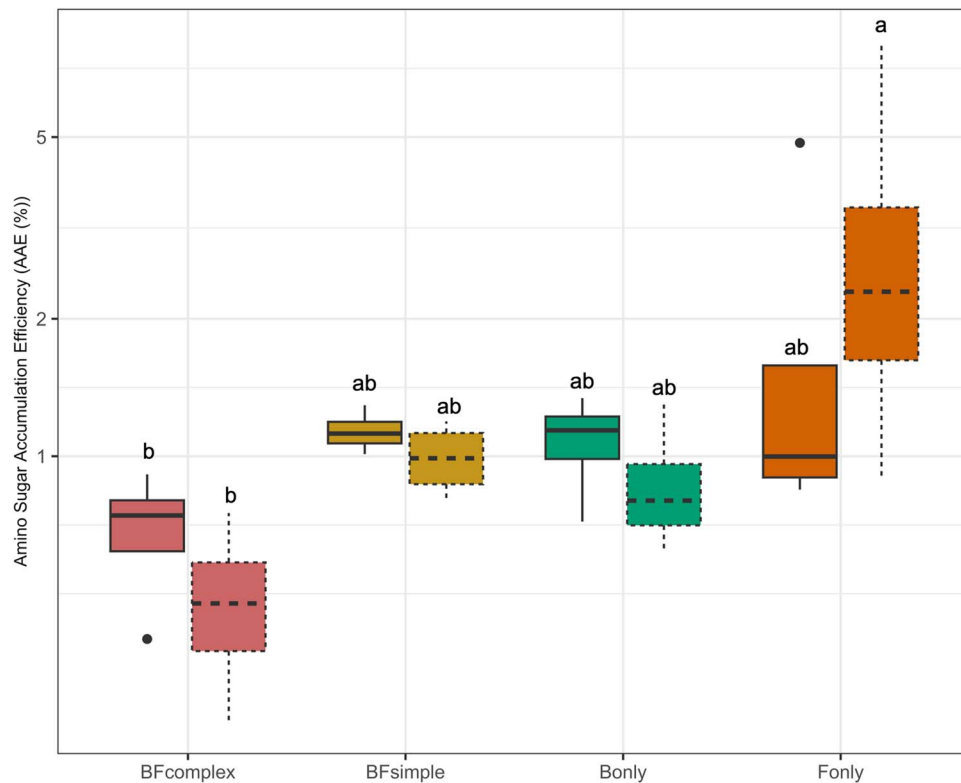


Figure 4. AAE (%). Amino sugar-C accumulated at the end of the FMSOC after four weeks of incubation taking into account the cumulative respiration produced by each specific microcosm: $AAE = (\text{AminoSugar-C} / (\text{AminoSugar-C} + \text{cumulative}(\text{CO}_2\text{-C})) * 100$. AAE is reported in percent (%). Boxplots showing the median, the interquartile range, the minimum and maximum values. Different letters indicate significant differences between communities.

glucosamine measurements correlated to the signal at higher temperatures (450–550°C) (Fig. 6A). Moreover, when evaluating the biological origin of the necromass (i.e. inoculation treatments) by the SOM thermal-signal it was the BF_{complex} treatment that generated the strongest thermal-stable signal (Fig. 6B). Despite F_{only} treatment showing the highest potential for necromass accumulation efficiency (Fig. 4), the SOM generated here correlated with a relatively narrow range of signal captured during ramped thermal pyrolysis at earlier temperatures compared to BF_{complex} (Fig. 6B). Furthermore, we observed a positive correlation between necromass of fungal and bacterial origin with soil aggregation (mesodensity fraction; Fig. 6D and Supplementary Fig. 6). Fungal necromass also correlated to the C-fraction (%) measured at higher temperatures (Fig. 6C). However, total amino sugars did not correlate with these parameters (Fig. 6E, F). Remaining substrate can be a limiting factor in such studies. To evaluate the remaining substrate we measured the TOC at the end of FMSOM and PSOM (Supplementary Fig. 2) and we estimated the remaining C taking into account C lost via respiration and the C allocated into biomass and necromass (Supplementary Fig. 3A), as well as the DOC at the end of the FSOM phase (Supplementary Fig. 3B). Our TOC measurements and estimates of C remaining suggest no difference between the different treatments while the DOC measurement suggest lower DOC values for the BF_{complex} treatment in comparison to other treatments. We also compared the FID/TOC signal for each specific soil and incubation phase in order to evaluate the differences in the thermal signals between the subsequent incubations (Supplementary Figs. 7–14). Overall, we observed a smaller FID/TOC signal in the PSOM phase compared to FMSOM phase showing that microorganisms further mineralized the SOM during the second phase of incubation.

Evaluating the dynamics and persistence of SOM of distinct microbial origin during the second incubation

We did not observe significant differences in the organic carbon remaining in these soils at the beginning of the second incubation phase (PSOM; Supplementary Fig. 2). When evaluating the respiration, growth and CUE in the PSOM phase, we explained a more significant fraction of the variation in the respiration dataset compared to growth or CUE (79%, 44% and 14% for respiration, growth and CUE, respectively) (Fig. 7). Moreover, we observed that amino sugars were negatively related to both respiration and growth measurements during the PSOM phase in these soils (Fig. 7). We also evaluated the relationship between amino sugar accumulated in the first phase of the experiment and the measurements observed in the PSOM phase for each individual community type (Fig. 8). The F_{only} community was the only treatment in which we did not observe any significant relationship between amino sugar produced during the FMSOM phase and cumulative respiration measured during 15 days, short-term respiration during CUE incubations and growth measurements. In all other treatments we observed a negative relationship between total amino sugar and some of the activity measurements with the exception of CUE, which was not significantly related to amino-sugar content in any of the community types (Fig. 8). As CUE is the compilation of both respiration and growth, the fact that CUE was not negatively correlated to amino sugar shows that the response of respiration and growth to amino sugar content was dissimilar. We also evaluated the relationship between TOC measured in the end of the first phase of the experiment and the measurements observed in the PSOM phase for each individual community type (Supplementary Fig. 5). Contrary to the

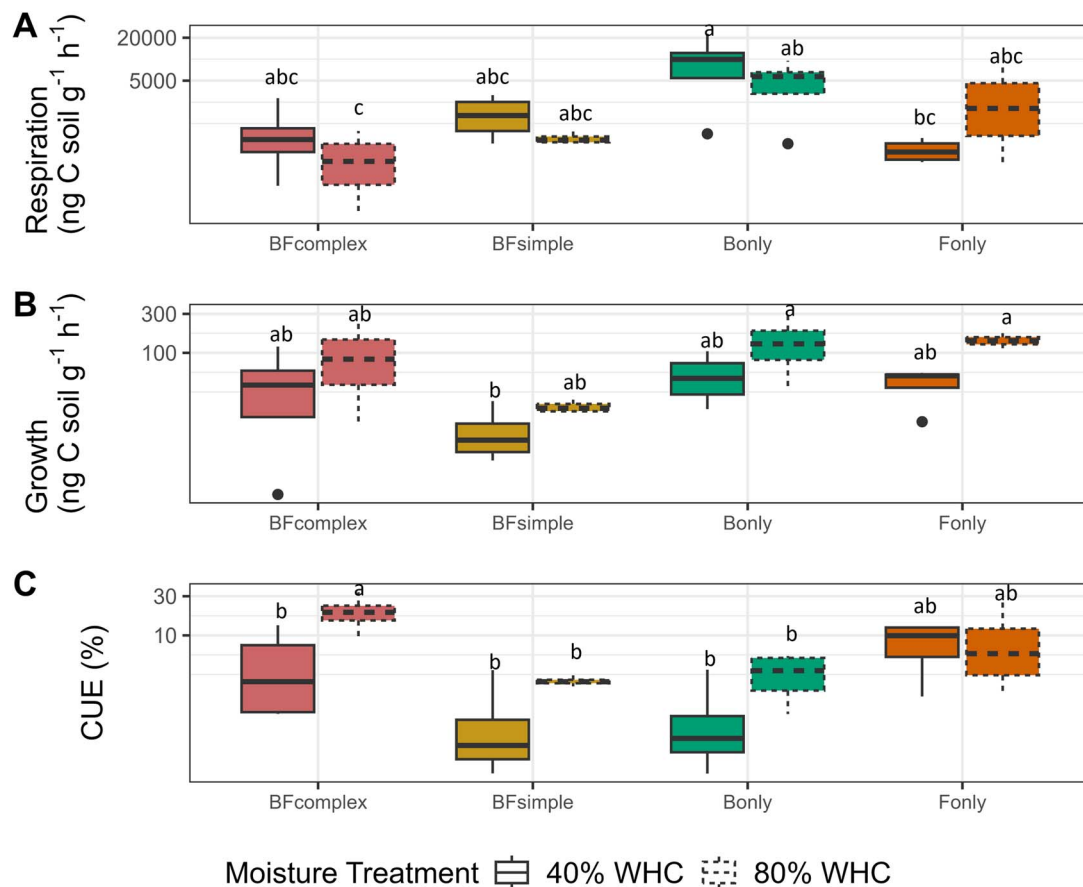


Figure 5. Carbon cycling processes measured at the end of the FMSOM incubation phase. Respiration (A), growth (B), and CUE (C) measured at the distinct communities under different moisture levels. Letters indicate significant differences between communities and moisture treatment.

relationships observed with amino-sugar, we observed an overall positive relationship between TOC and respiration and growth measurements (Supplementary Fig. 5).

Discussion

We manipulated microbial communities to control the necromass formation in model soils to test if necromass of different biological origins and formed under different abiotic conditions differ in its C thermal-signature and how this relates to its persistence in soils. We found evidence that fungal necromass is more thermally-stable than bacterial-necromass, when produced in soils with both bacteria and fungi present, suggesting the importance of microbial interactions for SOC stabilization. While we have an understanding that necromass is an important component of SOM [41, 51, 52] varying from 33% in soil forest to up to 62% in grassland soils [10], the role of necromass biological origin and dynamics within the microbial community for necromass persistence remains largely elusive. Our results show that while necromass chemistry was influenced by community-type, its total abundance was not, suggesting that the same amount of necromass (per unit of organic C) was formed in the distinct treatments. This allows us to evaluate the relative influence of necromass on SOM turnover and its influence on C-cycling processes in response to the distinct treatments [53]. As the remaining substrate in such studies can be a limiting factor, we aimed to inoculate communities of equivalent abundance by determining the CFUs of each inoculum, thereby reducing the bias of the initial inocula (methods section). We analyzed

the relationship between amino-sugars abundance and the FID signal captured during the pyrolysis phase of rock-aval to evaluate how the distinct biological origin and conditions of necromass formation would impact its persistence and SOM turnover. Overall, necromass content in the distinct treatments was positively related to the thermal-signal at higher temperatures and negatively related to the signal at lower temperatures, suggesting that microorganisms have consumed the labile substrate (captured at lower temperatures) and microbial growth and death (i.e. necromass production) contributes to a more thermal-stable SOM signature in these soils. The only exception was the muramic acid of bacterial origin which showed a weak but positive relationship with the signal at lower temperatures and negative relationship with the signal captured at higher temperatures. These results suggest that necromass of solely bacterial origin is less persistent in soil. Surprisingly, *Fonly* treatment necromass signal was not related to the thermal signal captured at higher temperatures while the necromass measured at the *BFcomplex* treatment was positively related to the signal at higher temperatures suggesting that concomitant growth of fungi and bacteria resulted in a more thermal-stable SOM signature. Buckeridge et al (2020) [54] observed that necromass chemistry influences necromass-necromass (i.e. organic-organic) interactions resulting in higher retention of bacterial necromass if yeast necromass was present. Our experimental design does not allow us to differentiate between fungal × bacterial interactions of active microorganisms or dead organic-organic necromass interactions as hypothesized previously by Buckeridge et al. [54] Nevertheless, our results show that necromass measured under

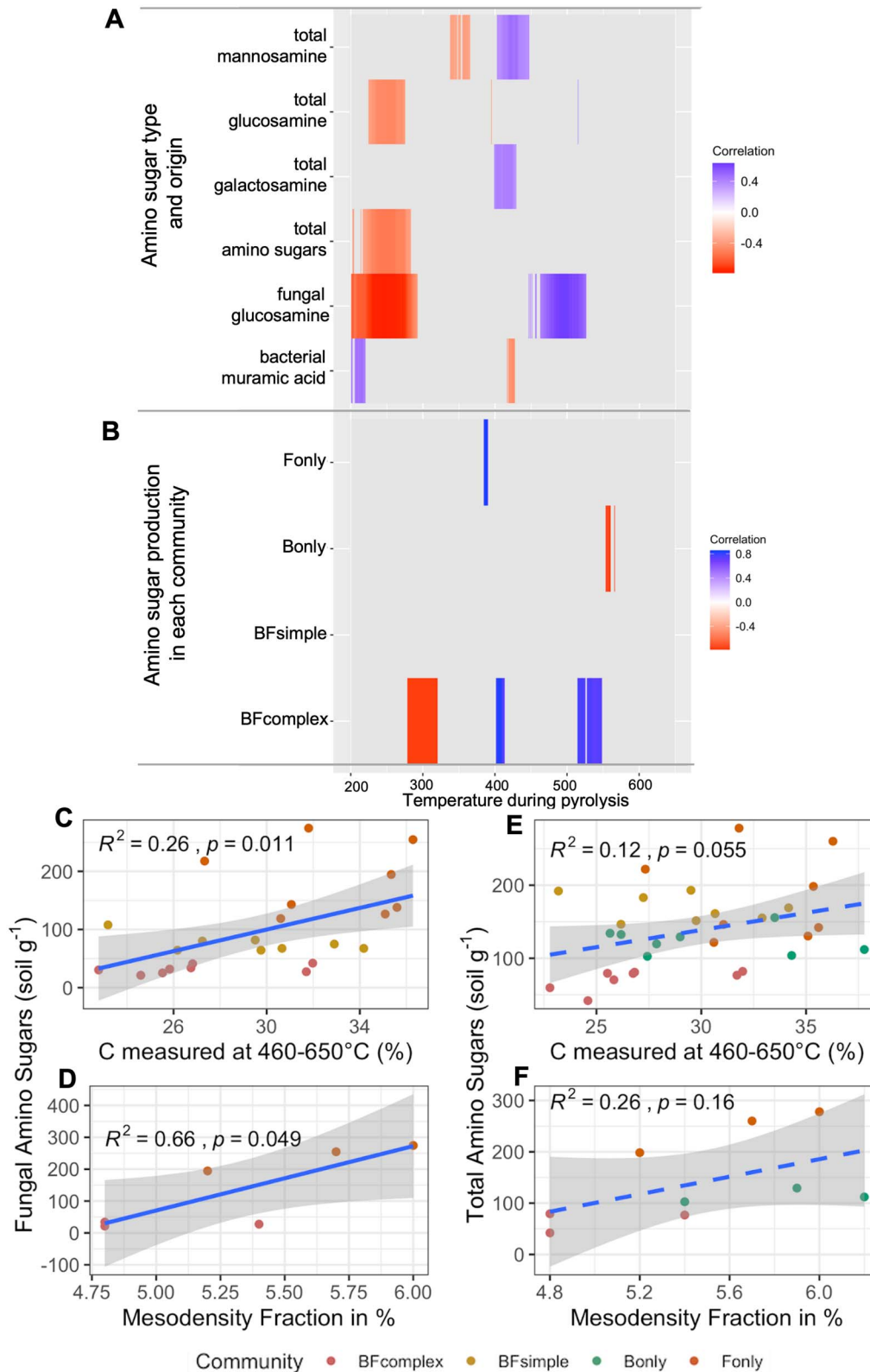


Figure 6. Relationship between the amino sugar residues, amino sugar biological origin according to incubation treatments and the SOM thermal-signal and the proportion of organo-mineral aggregate formed. Heat map of amino sugar residues and the FID signal captured during RE pyrolysis (A), relationship between amino sugars of fungal origin and the thermal-signal captured between 460–650°C (B), relationship between fungal amino sugars or total amino sugars (fungal and bacterial origin) and the thermal-signal captured between 460–650°C (C, E), and fungal amino sugars or total amino sugars and the mass proportion of meso-density aggregate formed (D, F).

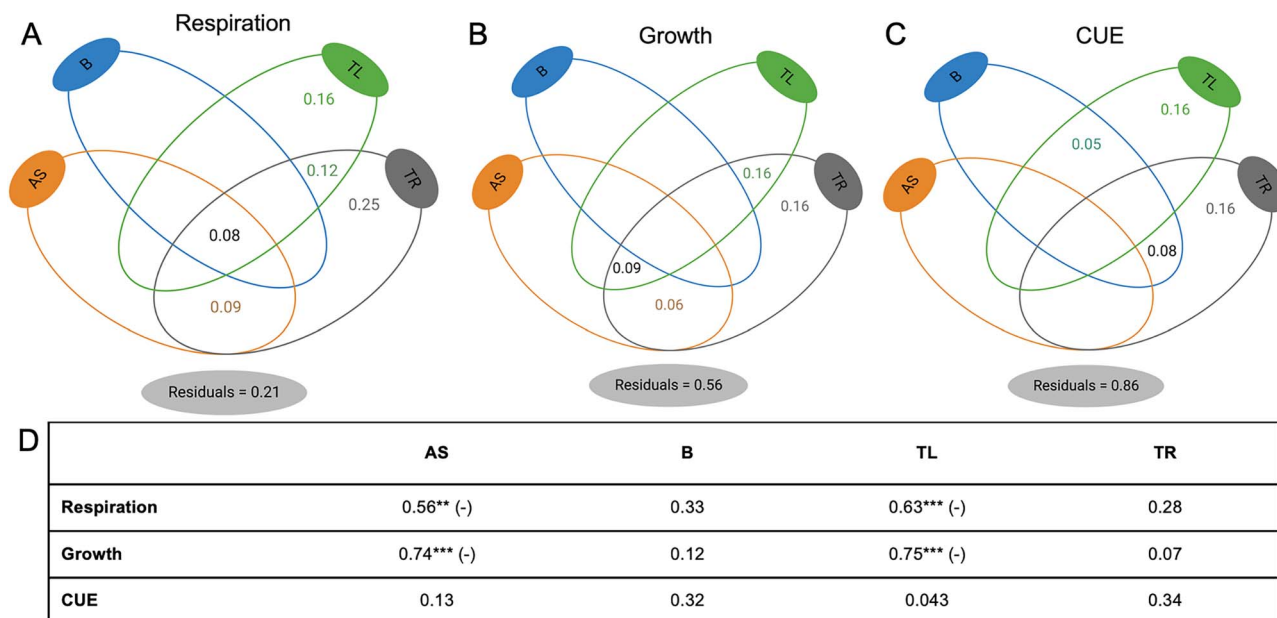


Figure 7. Variation partitioning of C cycling processes measured at PSOC in relation to the amino-sugar content, biomass and SOM thermal-signal measured at FMSOM. Variance was partitioned into amino-sugar (AS), microbial biomass (based on DNA yield; “B”), SOC thermal labile signal (“TL”), SOC thermal resistant signal (“TR”) and by combinations of these predictors. Variance partitioning of respiration (A), growth (B), and CUE (C). Table with correlation coefficients in addition to the directions of the correlations in case of positive (+) or negative (–) if significant (* $P < .05$, ** $P < .01$, *** $P < .001$) (D).

a more diverse microbial community (BF_{complex}) is related to a more thermally-stable SOM signature compared to the BF_{simple} treatment, which corroborates to our previous findings showing that fungal and bacterial Simpson diversity index was positively correlated to a SOM chemical signature requiring higher energy for its thermal-decomposition [17]. In a previous experiment [17], we observed that in the absence of fungi, bacterial communities produced a small pool of the extracellular enzyme to degrade chitin (i.e. N-acetylglucosaminidase) which is one of the components of the fungal cell wall and thus contributes to the necromass pool. Here we hypothesized that bacterial cells growing in the presence of fungi responded to the fungi presence by producing extracellular enzymes that resulted in further modifying and degrading the fungal cell wall which would increase necromass-turnover and the stabilization of the microbially-derived C. Accordingly, we observed the extracellular enzymatic pool to be the stronger driver of the SOM thermal-signature in a previous model soil experiment [17]. Alternatively, fungal biomass could also represent a source of organic nitrogen for community growth as N-limitation has been observed to be an important driver of microbial CUE [55].

Necromass persistence is likely to be linked with organo-mineral aggregation based on the fact that micro and submicron-sized aggregates (also called organo-mineral complexes) isolated from bulk soil by various physical fractionation techniques are characterized by strong microbial signatures such as low C:N ratio, high $\delta^{15}\text{N}$, amide-rich moiety, and by more negative $\Delta^{14}\text{C}$ (less input of modern C) [42]. We observed a positive relationship between fungal- and bacterial-derived amino-sugars and the soil mass measured at the mesodensity fraction, suggesting the relevance of microbial necromass for soil aggregation due to necromass-mineral interactions [30]. The agglomeration of fine mineral particles promoted by fungal necromass drives microaggregate formation and soil structure development [31]. These results suggest that microbial necromass stimulates soil

aggregation which is also known as a mechanism of OM occlusion and therefore protection from further decomposition within soil aggregates [32, 42]. Interestingly, we observed a positive relationship between fungal amino-sugar content in these soils and SOC pyrolyzed within 460–650°C, while the bacterial or total amino sugar content in these soils did not correlate to the more thermal stable C fraction (460–650°C). Future studies should further evaluate to which extent soil aggregation and necromass-origin influences the SOM thermal-signature and thermal-stability. A limitation of our study is that we used a single fungal strain (*Trichoderma koningii*), as other tested strains (i.e. *Mucor sp.* DSM 1222 and *Hypochnicium punctulatum* DSM 5040), did not grow in our model soil. Horsch et al. [23] showed that fungi contribution to mineral associated organic carbon (MAOC) was dependent on the specific traits of the arbuscular mycorrhizal fungal community. After a month of growth they showed that the Glomeraceae family and the mixed-family communities treatment contributed to MAOC formation while the Gigasporaceae family did not. Therefore, further studies should evaluate how more complex communities and their associated traits (e.g. growth rate, mycelial network structure, melanin and nitrogen contents) are associated with necromass formation and persistence in soil.

Previous studies have demonstrated that necromass accumulation efficiency depends on drivers such as clay content and mineralogy [41]. In this study our focus was on the influence of the microbial community, and therefore we used the same clay type and content across soils. We observed that the F_{only} at high moisture treatment respired on average less than the other treatments, resulting in a higher amino-sugar accumulation efficiency compared to BF_{complex} . We also conceived our experiment to evaluate if the distinct communities would exhibit contrasted CUE which could play a role in necromass formation. We showed that microbial respiration, growth and CUE measurements did not differ very strongly among community

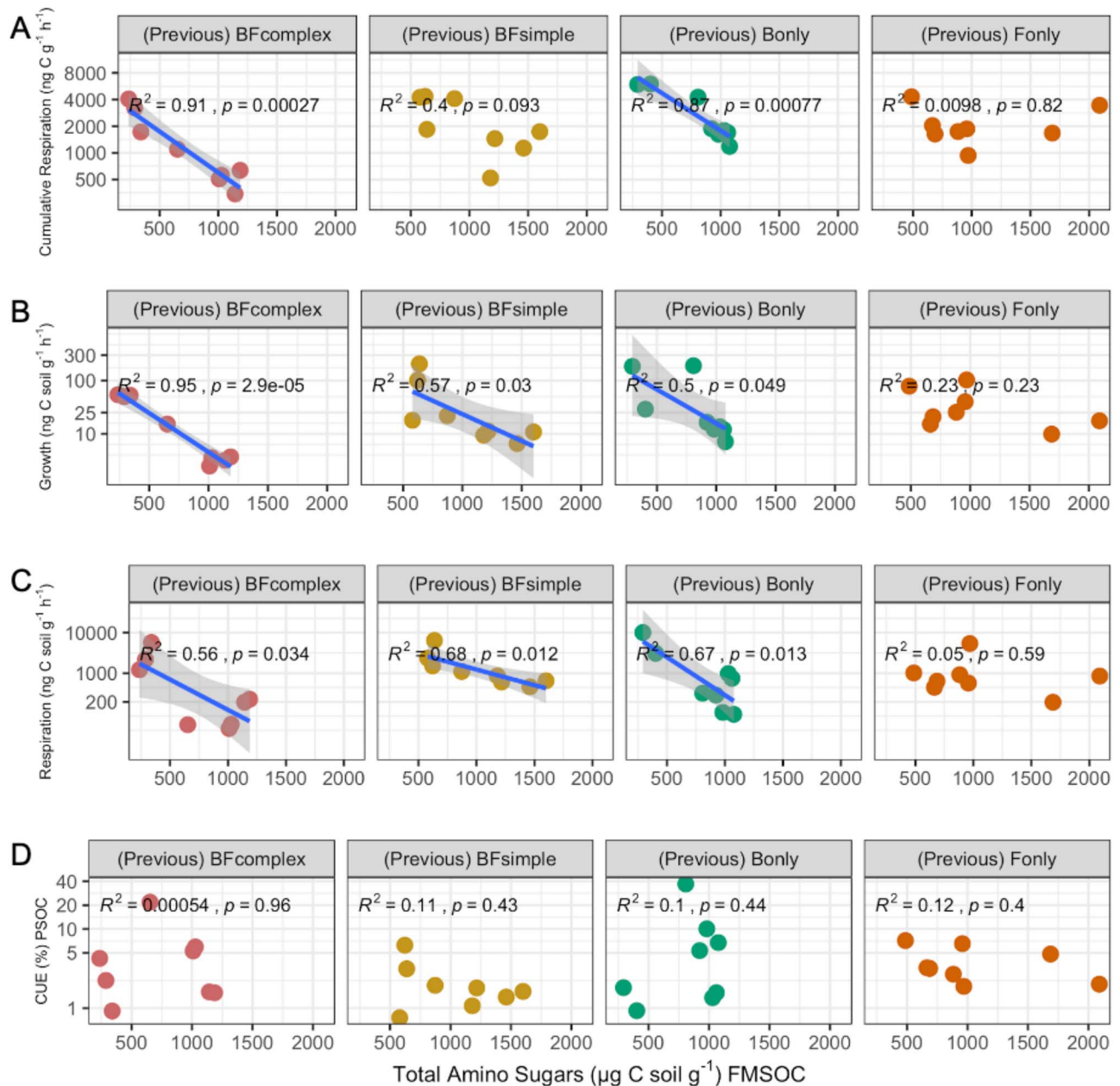


Figure 8. Relationship between total amino sugar measured at FMSOM and microbial C-cycling processes measured during PSOM for each community type. (A) Relationship between the microbial activity measured as cumulative respiration for two weeks during PSOM phase and the amino sugar-C content. (B) Relationship between growth measured at the end of PSOM and the amino sugar-C content. (C) Relationship between respiration measurement at the end of PSOM and the amino sugar C content. (D) Relationship between CUE measured at the end of PSOM and the amino sugar C content.

types or moisture treatments. However, abiotic drivers such as moisture are considered important drivers of microbial activities, but in our system moisture had a limited effect on necromass formation and microbial activity. This could be due to the fact that our lower moisture content did not exert physiological stress on the microorganisms. We did however observe a trend of higher microbial growth than respiration on more wet soils resulting in a tendency of higher CUE in wet compared to drier soils. Studies have observed distinct responses of CUE to drought, with some showing no significant effect [29], others reporting an increase in natural soils [56], and others finding a negative response in simple model soils [16]. While the discrepancy in these findings could be due to different methods used [57], fungal-dominated communities, are expected to be less sensitive to drier conditions [25, 58].

Our results show that the F_{only} treatment was less influenced by the two water moisture treatments, while communities containing bacteria showed a tendency to respond to moisture changes.

We observed that the necromass content formed in these soils was negatively related to respiration and growth suggesting that necromass constitutes a fraction of SOM not easily available to the microbial community re-inoculated in these soils as previously hypothesized [41, 51, 54]. While previous studies have highlighted the relevance of biotic-abiotic interactions for necromass retention (necromass-mineral interaction) [59], our results highlight that biotic interactions may also play a role in necromass formation and persistence. It has been recently hypothesized [60] – with some empirical support – that biotic interactions are underestimated drivers of CUE [61, 62]. We expected

to observe a negative relationship between CUE and necromass content, however we did not observe such relationship at PSOM in any of the treatments, although we recorded a negative relationship between amino-sugar content and both respiration and growth. While CUE is the composite measure of these other measures, we are hypothesizing that we did not observe a relationship with CUE because the catabolic and anabolic physiological responses of the microbial community to necromass presence in the soil were dissimilar. A possible explanation is that necromass content can differently affect growth and respiration. For example, microorganisms could allocate more C to non-growth metabolites such as extracellular enzymes [11] in an attempt to acquire substrate in an environment with decreasing C availability. This could suggest more “expensive growth” in response to physiological costs due to increases in the extracellular enzymatic pool and other costs related to acquisition of substrate and therefore we observe a de-coupling between respiration and growth as our current CUE measurements captures uniquely biomass increase [63] without measuring the C investment in non-growth.

Altogether our results show that the biological origin of necromass and the conditions under which necromass is formed (e.g. under fungal-bacterial co-existence) play a role in necromass persistence in soils. Model soils can be used as valuable tools to help us understand the mechanisms driving soil functioning [15–17, 41, 64], but our results cannot be extrapolated to natural soils without caution as microbial communities in soil are highly diverse and fungal and bacterial cells co-occur in large numbers. We also know that fungi are expected to dominate the first stages of plant biomass decomposition being followed by bacterial growth [20]. Our results showing the importance of co-occurrence of fungi and bacterial growth for necromass persistence could suggest the relevance of microbial succession for SOC stabilization in soils. Future studies should further investigate the role of microbial succession in SOM stabilization and whether there are potential context dependencies.

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Author contributions

Luiz A. Domeignoz-Horta and Selina Lepori conceptualized the experiment. Selina Lepori, Luiz A. Domeignoz-Horta, Nadja Rohner, and Viviana Loaiza performed the experiments. The amino-sugar measurements were performed at the Key Laboratory of Vegetation and Environmental Change at the Chinese Academy of Sciences (Beijing, China) by Xingqi Li under the supervision of Xiaojuan Feng. The density fractionation was performed at the Institute for Agro-Environmental Sciences (Ibaraki, Japan) by Rota Wagai and Daniel B. Nelson performed the stable isotope analyses. Selina Lepori analyzed the data under the supervision of Luiz A. Domeignoz-Horta. Selina Lepori and Luiz A. Domeignoz-Horta wrote the original draft of the manuscript and all authors contributed to revising and editing the manuscript.

Supplementary material

Supplementary material is available at ISME Communications online.

Conflicts of interest

The authors declare no conflict of interest.

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Data and code availability

The data and R code supporting the findings presented here are available from the corresponding author on request and from the GitHub https://github.com/Seleine/Soil_Microbes_C_Cycle/blob/3d913b412cd6e8f2ad22151f7d642e642360cfab/Master%20Thesis.

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