Soil Biology and Biochemistry 152 (2021) 108055

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

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

Soil aggregate-mediated microbial responses to long-term warming

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ARTICLE INFO

Keywords:
Aggregation and fractionation
Carbon and nutrient limitation
Microbe growth efficiency
Mineral-associated organic matter
Particulate organic matter decomposition
Substrate stoichiometry and accessibility
Necromass residues and sequestration
Beta-glucosidase and cellobiohydrolase
Phosphatase and N-acetyl-glucosaminidase
Phenol oxidase and peroxidase

ABSTRACT

Soil microbial carbon use efficiency (CUE) is a combination of growth and respiration, which may respond differently to climate change depending on physical protection of soil carbon (C) and its availability to microbes. In a mid-latitude hardwood forest in central Massachusetts, 27 years of soil warming (+5 °C) has resulted in C loss and altered soil organic matter (SOM) quality, yet the underlying mechanisms remain unclear. Here, we hypothesized that long-term warming reduces physical aggregate protection of SOM, microbial CUE, and its temperature sensitivity. Soil was separated into macroaggregate (250–2000 μm) and microaggregate (<250 μm) fractions, and CUE was measured with ^{18}O enriched water ($H_2^{18}O$) in samples incubated at 15 and 25 °C for 24 h. We found that long-term warming reduced soil C and nitrogen concentrations and extracellular enzyme activity in macroaggregates, but did not affect physical protection of SOM. Long-term warming showed little effect on CUE or microbial biomass turnover time because it reduced both growth and respiration. However, CUE was less temperature sensitive in macroaggregates from the warmed compared to the control plots. Our findings suggest that microbial thermal responses to long-term warming occur mostly in soil compartments where SOM is less physically protected and thus more vulnerable to microbial degradation.

1. Introduction

Soils play a central regulatory role in the global carbon (C) cycle (Davidson and Janssens, 2006), and soil C decomposition is under the control of microbes whose metabolisms are sensitive to temperature and substrate availability (Domeignoz-Horta et al., 2020; Frey et al., 2013; Hagerty et al., 2018; Liu et al., 2020). Short-term responses of soil organic matter (SOM) decomposition to warming may be driven by direct temperature effects on microbial physiology and labile substrates (Bradford et al., 2008; Schindlbacher et al., 2015; Tucker et al., 2013), while long-term responses may be driven by microbial thermal adaptation and changes in physical protection of SOM (Allison, 2014; Conant et al., 2011; Waring et al., 2020). However, the underlying mechanisms of soil C responses to climate warming remain unclear, because of unknown interactions between physical protection and microbial efficiency of SOM utilization.

Long-term warming may alter physical protection of SOM and affect substrate availability to microbes. SOM can be occluded within different sizes of aggregates or bound to mineral surfaces. In addition, SOM may be more vulnerable to microbial degradation in macroaggregates than in microaggregates due to less physical protection and higher substrate accessibility to microbes (Bandyopadhyay, 2020; Tian et al., 2015). Thus, macroaggregates and associated SOM appear to be susceptible to loss under warming (Wang et al., 2016) due to faster turnover rates than those for microaggregates (Balesdent et al., 2000). One reason for the disparity in warming responses between aggregates may be due to the different organization of SOM pools within them. Coarse particulate organic matter (cPOM) and fine POM (fPOM) are considered as unprotected SOM pools, while occluded POM (oPOM) and mineral-associated organic matter (MAOM or MOM) are considered as protected SOM pools

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https://doi.org/10.1016/j.soilbio.2020.108055
Received 9 July 2020; Received in revised form 17 October 2020; Accepted 21 October 2020
Available online 24 October 2020

that are resistant to microbial degradation (Sollins et al., 2006; von Lützow et al., 2007). However, several lines of evidence have shown that protected SOM fractions can also cause soil C loss over warming (Cheng et al., 2011; Pold et al., 2017; Schnecker et al., 2016), suggesting that biotic factors such as microbial physiological parameters are possibly responsible for warming-induced C loss.

Responses of microbial C use efficiency (CUE) to climate warming remain uncertain due to complex interactions among SOM quality, microbial activity, and microbial thermal responses. Chronic soil warming has been reported to increase the CUE of phenol but not that of glucose (Frey et al., 2013) or of SOM (Walker et al., 2018). Microbial CUE of SOM also shows different thermal responses, either decreasing or showing little response with increasing temperatures (Fuchslueger et al., 2019; Zheng et al., 2019). Biomass turnover time also shows variable responses, either decreasing or increasing with increasing temperatures (Hagerty et al., 2014; Walker et al., 2018; Zheng et al., 2019). Besides, increased enzyme activities under warming can also decrease CUE (Cates et al., 2019). These uncertainties of microbial responses can be caused by changes in physical protection and substrate accessibility to microbes, which have not been explored, especially for CUE of SOM after long-term soil warming.

Chronic warming in central Massachusetts (Melillo et al., 2002) has reduced soil C pools and shifted microbial communities (Bradford et al., 2008; DeAngelis et al., 2015; Pold et al., 2017). However, the interacting effects of long-term warming on microbial CUE and physical protection of SOM remain unclear. Here, we collected mineral soil (0–10 cm) from our experiment site where the soil has been experimentally warmed 5 °C above ambient temperature for 27 years (Melillo et al., 2017). We studied microbial processes in macroaggregates (250–2000 μm) and microaggregates (<250 μm). Given that our prior work has found large amounts of C loss, it is possible that long-term warming has reduced physical protection of SOM, and that most of the lost C was derived from less physically protected SOM, such as in macroaggregates rather than microaggregates. Hence, we hypothesized that long-term warming would 1) reduce physical protection of SOM and its availability to microbes, and 2) reduce microbial CUE and its temperature sensitivity.

2. Materials and methods

2.1. Experimental site and soil sampling

Soil samples were collected in October 2017 from the Soil Warming Study at the Harvard Forest Long-Term Ecological Research (LTER) site in Petersham, Massachusetts, USA (Peterjohn et al., 1994). Soil in the experimental plots (6 × 6 m) has been warmed 5 °C above ambient temperature since 1991 using buried cables at 10 cm depth, with disturbance control plots (identical to warmed plots except received no electric power) being used as the reference condition. The soil is a sandy loam Inceptisol, with a pH of 4.7, mean annual precipitation of 108 cm and mean annual temperature of 8 °C. Two cores (5.0 cm diameter, 0–10 cm depth) of mineral soil were collected from each plot following removal of the organic horizon, for a total of 16 cores (i.e., two treatments × four replicate plots per treatment level × 2 cores per plot). Fresh soil was sieved through a 2 mm mesh, and the bulk soil moisture was immediately measured by drying the soil (2.0 g) at 105 °C for 24 h.

2.2. Soil aggregate separation and density fractionation

To interpret responses of physical protection to long-term warming, relative abundances (aggregate mass:bulk soil mass) of microaggregates (<250 μm) and macroaggregates (250–2000 μm), and SOM pools (cPOM, fPOM, oPOM, MAOM) were measured. Bulk soil samples were allowed to dry under controlled conditions (4 °C) to reach a consistent moisture (10% gravimetric water content, based on target soil mass calculated from bulk soil moisture above after four days) to facilitate reproducible aggregate disruption and to minimize effects on microbial communities (Bach and Hofmockel, 2014). A 200 g soil sample (10% moisture) was placed on a 250 μm sieve and shaken horizontally by hand at a rate of 30 times min^{-1} for 2 min (Bach and Hofmockel, 2014; Tian et al., 2015). The aggregates were stored at 4 °C for soil physiochemical and microbiological analyses conducted within three weeks of sieving. Remaining aggregate fractions were stored at −20 °C and enzyme assays were conducted within eight months.

Density fractionation was conducted on subsamples (2.0 g) of each aggregate size class following previous protocols (Lajtha et al., 2014; Pold et al., 2017). Firstly, aggregates were soaked in deionized water in a specimen cup (120 mL) for 10 min, transferred to a 250 μm sieve that was submerged with 1.0 L deionized water in a plastic container, and shaken for 5 min (80 rpm) with 200 glass beads. Material passing through the sieve was transferred to a 53 μm mesh sieve, shaken for 2 min, after which the <53 μm fraction was transferred to a 1.0 L bottle, settled for 6 h, and filtered (0.7 μm). All fractions (>250, 53–250, and <53 μm) were dried overnight (65 °C) and weighed.

On the next day, 10 ml of sodium polytungstate (SPT; 1.85 g ml^{-1}) was added to the >250 μm aggregates in a 50 ml falcon tube and centrifuged at 2000×*g* for 30 min. The supernatant was filtered (0.7 μm) and collected as cPOM. Another 10 ml SPT was added to a 50 ml tube with the 53–250 μm fraction, centrifuged for 30 min, and the supernatant was filtered and collected as fPOM. The pellets were transferred to a 50 ml tube with 10 ml SPT, shaken at 100 rpm for 2 h with 10 glass beads and centrifuged for 30 min. The supernatant was filtered and collected as oPOM, while the pellets were filtered and collected as MAOM (Pold et al., 2017). The density of the SPT solution was checked between samples to maintain rinse-through density within ±0.01 g mL^{-1}. All density fractions were dried at 65 °C overnight, followed by the soil C and nitrogen (N) analyses using the dry combustion method (Liu et al., 2017, 2020).

2.3. Soil physical and chemical analyses

Soil water holding capacity (WHC) was determined for the aggregate fractions by allowing thoroughly wetted soil (2.0 g) to drain in a Whatman #3 filter for 0.5 h for macroaggregates and 1.5 h for microaggregates (durations differ due to different WHC values and water percolation rates through pore space between macroaggregates and microaggregates). The saturated soil was weighed and dried at 105 °C for 24 h. Another 2.0 g soil of each aggregate size class was air dried and ground to the fine powder (20–40 mg) for soil C and N analyses (Liu et al., 2017, 2020).

2.4. Microbiological analyses

Soil microbial biomass C (MBC) of aggregate fractions was determined using the chloroform fumigation extraction method (Vance et al., 1987) with some modifications (Liu et al., 2017, 2020): A 2.0 g subsample was extracted with 10 ml K_2SO_4 solution (0.5 M) and filtered (Whatman #3). The filtrate was diluted with K_2SO_4 at a 1:2 ratio and dissolved organic C (DOC) was measured by a TOC-L analyzer (Shimadzu, Kyoto, Japan). Another 2.0 g subsample was fumigated for 24 h in a sealed desiccator and analyzed following the same procedure. MBC was calculated as the difference in DOC before and after fumigation corrected by 0.45 (Finley et al., 2018).

Hydrolytic and oxidative enzyme assays were performed following previous protocols (German et al., 2011; Liu et al., 2020). The hydrolytic enzymes acid phosphatase (AP), N-acetyl-glucosaminidase (NAG), β-glucosidase (BG) and cellobiohydrolase (CBH) were assayed using fluorometric substrates (4-Methylumbelliferyl-phosphate, N-acetyl-β-D-glucosaminide, β-D-glucopyranoside and β-D-cellobioside), and oxidative enzymes peroxidase (PER) and phenol oxidase (POX) were assayed using L-3,4-dihydroxyphenylalanine (L-DOPA) with and without H_2O_2 (0.03%). Soil aggregates were taken from the freezer, immediately weighed (~1.0 g), and soil slurries were prepared by

homogenizing the aggregates in a Waring Commercial Blender with 125 ml of sodium acetate buffer (SAB; 50 mM; pH = 4.7) for 2 min, and 200 μl aliquots were transferred to a 96-well plate. 50 μl of substrate solution was added to each experimental well. Blank wells within each plate consisted of 250 μl of 50 mM SAB. Reference wells consisted of 200 μl of SAB and 50 μl of 100 μM 4-Methylumbelliferone (MUB) standard. Negative control wells consisted of 200 μl of SAB and 50 μl of substrate solution. Homogenate controls received 50 μl of SAB, quench controls received 50 μl of MUB standard, and assay wells received 50 μl of substrate solution after adding 200 μl of soil slurry to sample wells. Plates were incubated for 2–4 h in the dark at room temperature (~20 °C) before being measured for fluorescence (365/450 nm). Fluorescent enzyme activity was calculated as described by Liu et al. (2020):

$$\text{Activity}\ \left(\text{nmol g}^{-1}\ \text{h}^{-1}\right) = \frac{\textit{Net fluor.} \times \textit{Buffer vol (ml)}}{\textit{Emission coeff.} \times \textit{Homogenate vol (ml)} \times \textit{Time (h)} \times \textit{Soil (g)}} \quad (1)$$

where,

$$\text{Net fluor.} = \left(\frac{\textit{Assay} - \textit{Homogenate control}}{\textit{Quench coeff.}}\right) - \text{Substrate control} \quad (2)$$

$$\text{Emission coeff.}\ \left(\text{Fluor. nmol}^{-1}\right) = \frac{\textit{Standard fluor.}}{\textit{Standard concentration}\ \left(\textit{nmol ml}^{-1}\right) \times \textit{Standard vol (ml)}} \quad (3)$$

$$\text{Quench coeff.}\ \left(\text{Fluor. nmol}^{-1}\right) = \frac{\textit{Quench control} - \textit{Homogenate control}}{\textit{Standard Fluor.}} \quad (4)$$

For oxidative enzyme assays, 50 μL substrate solution (L-DOPA) was added to each well, with PER wells receiving an additional 10 μL of H_2O_2 (0.03%). Both assays were incubated for 24 h at room temperature and measured for absorbance (460 nm). With an extinction coefficient of 7.9 (Bach et al., 2013), the POX activity was calculated as below, and the PER activity was the difference between H_2O_2-amended and POX wells (Liu et al., 2020).

$$\text{Net Absorbance} = \text{Assay} - \text{Homogenate control} - \text{Substrate control} \quad (5)$$

$$\text{Activity}\left(\mu\text{molg}^{-1}\ \text{h}^{-1}\right) = \frac{\textit{Net absorbance} \times \textit{Buffer vol (ml)}}{\textit{Extinction coeff.} \times \textit{Homogenate vol (ml)} \times \textit{Time (h)} \times \textit{Soil (g)}} \quad (6)$$

2.5. Laboratory incubation experiment

Microbial CUE was determined using a substrate-independent, ^{18}O-isotope labeling method ($H_2^{18}O$) that quantifies the amount of ^{18}O incorporated into microbial DNA during a brief laboratory incubation. This technique does not capture extracellular enzyme activities or extracellular polymeric substances, but can more accurately estimate community growth rate and turnover time compared to substrate-related CUE methods (Geyer et al., 2019; Spohn et al., 2016). The experiment had four field replicates with three treatment factors: long-term soil warming (control and warmed), aggregate size (macroaggregates, 250–2000 μm; microaggregates, <250 μm), and incubation temperature (15 and 25 °C). Lab incubation temperatures were chosen to represent typical field soil temperatures during the spring and summer seasons (Bradford et al., 2008; Frey et al., 2013).

A six-day pre-incubation was conducted by transferring 10.0 g (dry-weight equivalent) aggregates to 50 ml falcon tubes at 15 or 25 °C at 40% WHC. These tubes were open for 10 min daily with moisture adjustment to avoid excessive CO_2 accumulation and anoxic conditions (Liu et al., 2017, 2020). Following the pre-incubation, 750 mg soil was transferred to three microtubes (2 mL; 250 mg soil per tube as technical replicates) and sealed in a 27 ml glass tube with a rubber stopper and crimp to obtain sufficient microbial respiration above detection level. Half of the samples was amended with ^{18}O natural abundance water and the other half with ^{18}O enriched water (97 atm%) to reach a final 20.0 atm% ^{18}O-labeled soil water (Spohn et al., 2016). The incubation was conducted for 24 h at 60% WHC.

Gas samples were taken from the tube head space at the beginning and end of incubation, and the CO_2 concentration was measured using an infrared gas analyzer, with CO_2-free air being used to flush the syringe between samples. The soil samples were frozen immediately at −80 °C after gas sampling. DNA was extracted with a Qiagen DNeasy PowerSoil Kit following the manufacturers' protocol, with modifications to improve DNA yield (Geyer et al., 2019). DNA was quality checked (NanoDrop), quantified (PicoGreen assay), dried in silver capsules at 60 °C for 24 h, and shipped to the UC Davis Stable Isotope Facility for ^{18}O analysis, using an Elementar PyroCube (Hanau, Germany) interfaced to an Isoprime VisION (Stockport, UK).

2.6. Calculations and statistical analyses

Microbial respiration (R; μg C g^{-1} soil h^{-1}) was calculated based on CO_2 fluxes produced during the incubation period. Microbial growth (G; μg C g^{-1} soil h^{-1}) was the flux of C from SOM allocated to biomass production, which was estimated from newly formed DNA by specific MBC:DNA ratios of individual samples. Such newly formed DNA was

calculated from excess enriched ^{18}O-labeled against ^{18}O-natural-abundance DNA samples using a conversion factor of 31.9% (assuming 31.9% of DNA is from oxygen), which was well in the range of conversion factors (31.0–34.3%) reported in literature (Geyer et al., 2019; Papp et al., 2018; Qu et al., 2020; Spohn et al., 2016). The final excess ^{18}O signature of DNA was calculated using an isotope mass balance equation (Liu et al., 2017, 2020). Microbial organic C uptake (μg C g^{-1} soil h^{-1}) was estimated as the sum of respiration and growth. Mass-specific respiration (R_m), growth (G_m), and organic C uptake (U_m) were calculated by dividing MBC determined at the beginning of incubation. Microbial CUE and turnover time were calculated based on the steady-state assumption (Hagerty et al., 2014; Liu et al., 2020; Spohn et al., 2016):

$$\text{CUE}\ (\%) = \frac{Growth}{Growth + Respiration} \times 100\ \% \quad (7)$$

$$\text{Turnover time}\ (\text{day}) = \frac{MBC}{Growth \times 24\ h} \quad (8)$$

Temperature sensitivity (Q_{10}) was used in this study to describe changes of microbial activity and DOC from 15 to 25 °C (Plante et al., 2009; Schindlbacher et al., 2015). To facilitate interpretation of Q_{10} values, especially when $Q_{10} < 1.0$ (commonly observed for CUE), we show the log-transformed Q_{10} values (log-Q_{10}), such that log-Q_{10} of zero indicates no temperature sensitivity, log-Q_{10} of 0.30 is a doubling of the rate and log-Q_{10} of −0.30 is half of the rate.

Data were checked for parametric assumptions using residual plots and Shapiro-Wilk and Levene tests, and were log-transformed to meet the normality assumption as needed. Treatment effects were tested using the repeated measures ANOVA (field plot as a random effect), followed by a posthoc test for multiple comparisons (*glht*), with p-values adjusted (Benjamini-Hochberg method). Variance of response variables explained by treatment factors was obtained from partitioning of sum of squares from repeated ANOVA analyses (Liu et al., 2020). Microbial processes and DOC were predicted using linear mixed effects models (REML method; backwards selection). Model structures were tested for alternate variance structures with field plots as a random effect, and models were selected based on the AIC criteria. All analyses were performed in R (version 4.0; R Core Team, 2020), using the packages *ggplot2, multcomp,* and *nlme* (Hothorn et al., 2008; Pinheiro et al., 2020; Wickham, 2016).

3. Results

3.1. Long-term warming reduced substrate availability and enzyme activity but not physical protection of SOM

Long-term warming reduced SOC and soil N in macroaggregates but not in microaggregates (Fig. 1a). However, warming reduced DOC in microaggregates, and DOC was greater in microaggregates than in macroaggregates (Fig. S1). We also found that DOC was best predicted by long-term warming and aggregate size (Table 1).

Hydrolytic enzyme activities decreased with long-term warming, while oxidative enzyme activities showed little response (Fig. 1b). On average, the activities of acid phosphatase (AP), β-glucosidase (BG), cellobiohydrolase (CBH) and N-acetyl-glucosaminidase (NAG) declined more than 70% in macroaggregates from the warmed than those from the control plots (Fig. 1b). Warming also reduced microbial biomass specific activity of BG and CBH in macroaggregates (Fig. S2). Activity of peroxidase (PER) and phenol oxidase (POX) was not affected by warming. Stronger potential microbial C limitation than N and phosphorus limitation was evidenced by increased ratios of (PER + POX):(BG + CBH), (PER + POX):NAG and (PER + POX):AP in macroaggregates of warmed compared to control plots (Table S1).

Long-term warming showed little effect on aggregate size distribution or SOM density fractions (Figs. 1a and S3). The relative soil mass abundances of protected SOM pools (oPOM and MAOM) were not affected by warming (Fig. S3). However the relative abundance of fPOM in microaggregates was lower in the warmed than control plots. Warming reduced soil C and N concentrations in fPOM, which were offset by a trend towards increasing soil C and N concentrations in MAOM, especially in microaggregates. Compared to macroaggregates, microaggregates had greater fractions of MAOM and greater silt/clay content, but had lower fractions of cPOM (Fig. S3 and Table S1). Most of the soil C and N stocks in macroaggregates was from cPOM but most in microaggregates was from MAOM.

3.2. Warming reduced microbial growth and respiration but not CUE

The efficiency with which microbes utilize SOM showed little response to long-term warming, because of simultaneous decrease in microbial growth and respiration (Fig. 2). However, long-term warming

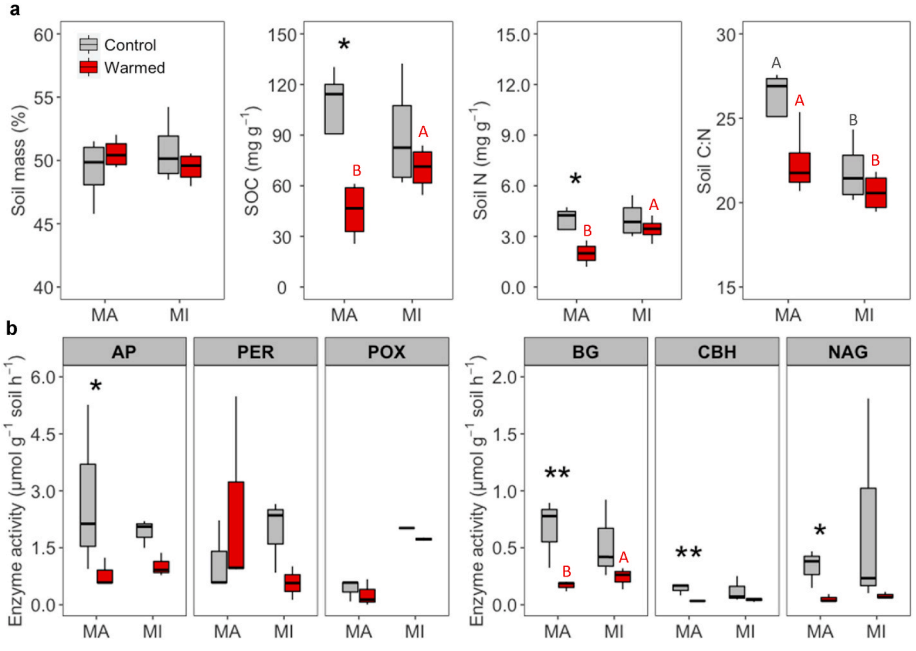

Fig. 1. Soil structure, substrate availability, and microbial responses to long-term warming. **a.** Relative abundance of aggregates, soil C, N, and C:N ratio. **b.** Extracellular enzyme activity. MA, macroaggregates (250–2000 μm); MI, microaggregates <250 μm); AP, acid phosphatase; BG, β-glucosidase; CBH, cellobiohydrolase; NAG, N-acetyl-glucosaminidase; PER, peroxidase; POX, polyphenol oxidase. * and ** indicate significant warming effects ($P < 0.05$ and 0.01). Different letters indicate significant differences ($p < 0.05$) between macroaggregates and microaggregates for either control (grey) or warmed (red) plots. Boxplot shows median, first and third quartiles and whiskers are maximum and minimum values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Dissolved organic C (DOC) and microbial physiology components predicted using linear mixed effects models with experimental effects as inputs.

Parameter	Model structure	Model statistics	Fitted values	P-values
DOC (μg C g^{-1} soil)	DOC ~ Agg + Warm	F = 2789	Intercept = 347	<0.0001
		$P < 0.0001$	Aggregate (micro) = +158	<0.0001
		df = 29	Warm (warmed) = −68	<0.0001
CUE (%)	CUE ~ Temp	F = 946	Intercept = 42.2	<0.0001
		$P < 0.0001$	Temp = −1.39	<0.0001
		df = 30		
Respiration (R) (μg C g^{-1} soil h^{-1})	R ~ Warm + Temp	F = 113	Intercept = 1.68	<0.0001
		$P < 0.0001$	Warm (warmed) = −0.81	<0.0001
		df = 29	Temp = +0.10	<0.0001
Growth (G) (μg C g^{-1} soil h^{-1})	G ~ Warm	F = 301	Intercept = 1.20	<0.0001
		$P < 0.0001$	Warm (warmed) = −0.49	<0.0001
		df = 30		
R_m (μg C g^{-1} C_{mic} h^{-1})	R_m ~ Agg + Temp	F = 589	Intercept = 2.85	<0.0001
		$P < 0.0001$	Aggregate (micro) = −1.28	<0.0001
		df = 29	Temp = +0.15	<0.0001
Turnover time (T) (day)	T ~ Warm + Agg	F = 689	Intercept = 20.8	<0.0001
		$P < 0.0001$	Aggregate (micro) = +13.1	<0.0001
		df = 29	Warm (warmed) = +6.3	0.0115

Note: CUE, C use efficiency; C_{mic}, microbial biomass C; R_m, mass-specific respiration; Warm, long-term warming treatment, with levels of warmed or control; Temp, incubation temperature 15 °C (normalized level) or 25 °C; Agg, aggregate size (macroaggregates and microaggregates); df, degrees of freedom. Model structures are linear mixed effect models using the REML method obtained using backwards selection, with field plots being included as a random effect.

did not affect mass-specific respiration, mass-specific growth, or turnover time. CUE was lower at 25 °C than at 15 °C, driven by greater respiration but not growth. We found that CUE was best predicted by incubation temperature only, but the components of CUE (i.e., growth and respiration) had different predictors (Table 1): growth was best predicted by long-term warming and respiration was best predicted by long-term warming and incubation temperature. Mass-specific respiration was best predicted by aggregate size and incubation temperature. Warming tended to reduce MBC and organic C uptake, and microaggregates had greater MBC than macroaggregates (Figs. S1 and S4). CUE had significant negative correlations with mass-specific respiration but had weak positive correlations with mass-specific growth (Fig. S5).

3.3. Temperature sensitivity of microbial physiology and substrate availability

Long-term warming reduced the temperature sensitivity of microbial processes but not the temperature sensitivity of DOC concentration. CUE was less temperature sensitive in macroaggregates from warmed compared to control plots, driven mostly by lower temperature sensitivity of respiration (Table 2). Warming also reduced the temperature sensitivity of mass-specific respiration but showed little effect on the

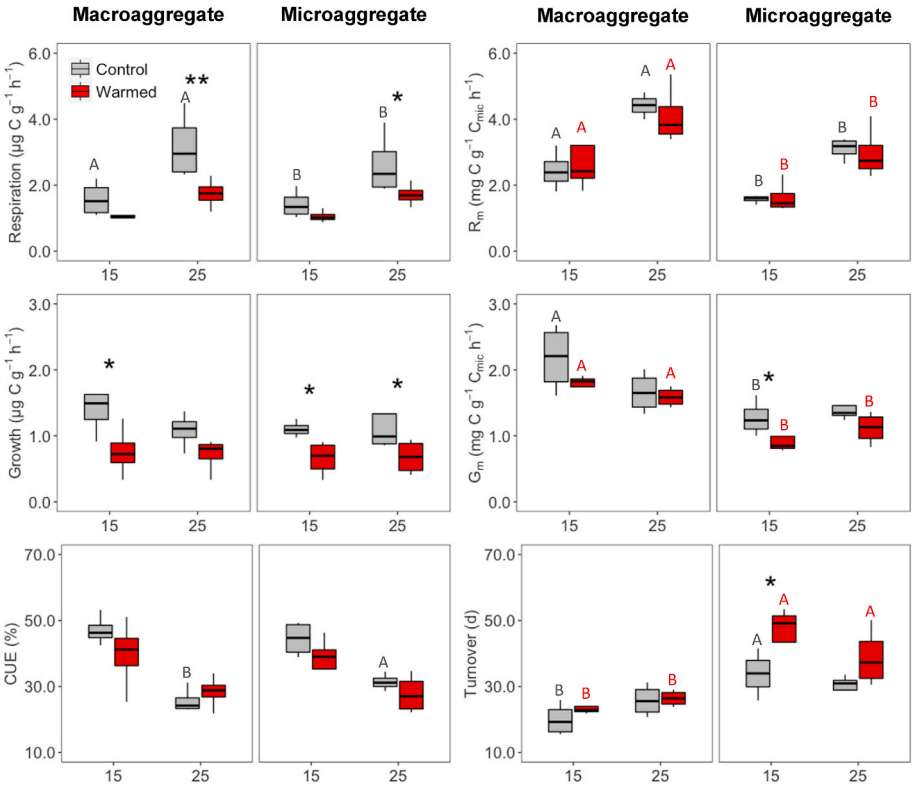

Fig. 2. Microbial responses to long-term warming as mediated by aggregate size and lab incubation temperature (15 and 25 °C). CUE, C use efficiency; R_m, mass-specific respiration; G_m, mass-specific growth. * and ** indicate significant warming effects ($P < 0.05$ and 0.01). Different letters indicate significant differences ($p < 0.05$) between macroaggregates and microaggregates for either control (grey) or warmed (red) plots at 15 °C or 25 °C. Boxplot shows median, first and third quartiles and whiskers are maximum and minimum values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Temperature sensitivity (Q_{10}; log-transformed) of dissolved organic C (DOC) and microbial physiology components over chronic warming.

Parameter	Macroaggregate (250–2000 μm)		Microaggregate (<250 μm)	
	Control	Warmed	Control	Warmed
DOC	0.03 (A)	0.03	0.01 (B)	0.02
Respiration	**0.31**	**0.20**	0.26	0.21
Growth	−0.11	−0.01	0.02	0.02
R_m	**0.30**	**0.17**	0.29	0.26
Turnover time	0.11 (A)	0.05	−0.05 (B)	−0.07
CUE	**−0.26** (B)	**−0.14**	−0.15 (A)	−0.13

Note: R_m, mass-specific respiration; CUE, C use efficiency. Bold numbers indicate differences between control and warmed plots, and different letters indicate differences between macroaggregates and microaggregates in control or warmed plot ($P < 0.05$; two-way repeated ANOVA). Log-Q_{10} of 0, 0.30, and −0.30 indicate no temperature sensitivity, doubling of the rate and half of the rate, respectively.

temperature sensitivity of turnover time, organic C uptake, DOC, or MBC (Tables 2 and S2).

Temperature sensitivity of microbial physiology and substrate availability was significantly different between macroaggregates and microaggregates in the control plots. CUE, turnover time, mass-specific growth, and DOC were more temperature sensitive in macroaggregates than in microaggregates derived from the control plots (Tables 2 and S2), but microbial temperature sensitivity did not differ between macroaggregates and microaggregates in warmed plots.

3.4. Environmental drivers of microbial physiology and substrate availability

Microbial physiology and substrate availability were driven by different environmental factors (Fig. 3a). For CUE, incubation temperature was the single best predictor (Table 1) and explained more than 50% of the variance (Fig. 3a). Respiration had a similar profile of drivers as CUE, except it showed a more pronounced response to long-term warming compared to CUE. Incubation temperature and aggregate size were also the best predictors for mass-specific respiration and explained more than 60% of its variance.

Long-term warming was the single best predictor of growth and explained more than 40% of the variance (Fig. 3a and Table 1). Turnover time was driven and best predicted by aggregate size and long-term warming. Though turnover time and MBC were not highly correlated ($r = 0.27$, Fig. S6), both were mainly explained by long-term warming and aggregate size (Figs. 3 and S7). Aggregate size explained most of the variance in mass-specific growth, organic C uptake, and DOC, with MBC and DOC sharing a reasonable correlation ($r = 0.68$, $P < 0.001$) (Figs. S6 and S7). More similar to CUE and respiration, growth:respiration ratio was driven mostly by the incubation temperature.

The main drivers for the temperature sensitivity of CUE were long-term warming and aggregate size (Fig. 3b). Temperature sensitivity of respiration and mass-specific respiration was driven mainly by long-term warming, while other microbial activities (e.g., growth, turnover time) and DOC were driven by aggregate size (Figs. 3 and S7).

4. Discussion

In this study, we evaluated how nearly three decades of warming in a temperate forest has impacted soil physical protection of SOM and microbial growth efficiency, both crucial components for soil C cycling. We hypothesized that soils subjected to long-term warming would reduce physical protection (as characterized by smaller proportions of macroaggregates and unprotected SOM pools (cPOM, fPOM) based on soil mass), but we found that distributions of aggregates and density fractions were mostly unchanged. SOC, soil N, and enzyme activity were more depleted in the warmed compared to control soils, particularly in macroaggregates where more vulnerable soil C pools reside. We also hypothesized that warming reduces microbial efficiency of utilizing SOM and reduces turnover time. However, warming did not affect CUE or turnover time, but reduced respiration and growth simultaneously, with respiration being driven by long-term warming and incubation temperature and growth being driven by long-term warming and aggregate size. Finally, we hypothesized that long-term warming would reduce microbial temperature sensitivity of growth efficiency. We found that temperature sensitivity of CUE decreased with long-term warming, driven by reduced temperature sensitivity of respiration, more so in macroaggregates compared to microaggregates.

4.1. Soil structure affects substrate availability in the microbial response to warming

Long-term warming reduced soil C and N concentrations in macroaggregates, where substrates were less physically protected than microaggregates (Cambardella and Elliott, 1993; Six et al., 2000). Warming also reduced DOC concentration, supporting the idea that chronic warming has accelerated microbial decomposition of labile C (Melillo et al., 2017; Pold et al., 2017). Consistent with prior findings (Pold et al., 2017), we found that long-term warming showed little effect on soil aggregation or SOM fractions. However, warming reduced the amounts of SOC and N in fPOM and tended to increase them in MAOM (Poeplau et al., 2020), possibly due to: 1) microbial activities and accessibility to substrates were lower in MAOM, thus reducing SOM degradation in the warmed compared to control plots; 2) by contrast, microbes in the unprotected fractions may have access to more substrates and increased the C loss from these fractions (relative to C in MAOM); and 3) long-term warming might have caused large amounts of microbial residuals (i.e., necromass), which could also raise the C content in MAOM. Our findings reveal that macroaggregates are more vulnerable, losing more than 70% of soil C and N concentrations than microaggregates, and suggest the crucial role of

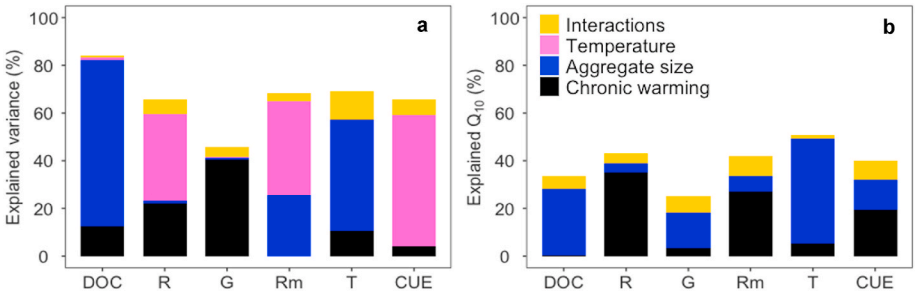

Fig. 3. Variance of dissolved organic C (DOC) and microbial activities (a) and their temperature sensitivity (b; Q_{10}) explained by treatment factors (Chronic warming, control and warmed; Aggregate size, macroaggregates (250–2000 μm) and microaggregates (<250 μm); Temperature, incubation temperature at 15 and 25 °C). R, respiration; G, growth; R_m:, mass-specific respiration; T, turnover time; CUE, C use efficiency.

microaggregate-protected SOM pools (e.g., MAOM) for soil C stabilization with long-term warming.

Our results suggest potential soil C and nutrient limitation due to reduced substrate availability after long-term warming. This is evidenced by increased ratios of oxidative enzymes relative to hydrolytic enzymes, and a trend for decreasing NAG:AP ratio with chronic warming (Table S1), especially in macroaggregates. Prior studies showed that microbes under labile SOM limitation were less competitive in acquiring C than other nutrients (Soong et al., 2018), thus increased functional potential for degrading complex C (Pold et al., 2016) and may offset some of the reduction in microbial biomass (Frey et al., 2008; Pold et al., 2017). By contrast, microbes adapted to reduced substrate availability in warmed soils increased mass-specific glycosidic enzyme activity (Pold et al., 2017). These differences may be due to the fact that soils between various studies were collected in different field seasons and soil depths when microbial activities had larger variations and greater seasonal effects than treatment effects. Together, these results demonstrate the dynamic nature of microbial responses to warming through time.

Climate warming can accelerate changes in accessibility and decomposition of SOM without changes to physical protection parameters (Conant et al., 2011). We found that long-term warming did not affect relative abundances of soil aggregates or density fractions. However, microaggregates had greater fractions of silt/clay and MAOM, suggesting that warming changed some of the SOM substrates without changing the overall soil structure (Poeplau et al., 2017). This is consistent with prior studies that distributions of aggregates (Cheng et al., 2011) or density fractions (Pold et al., 2017) were not affected by chronic warming. However, studies in subarctic grassland and forest ecosystems showed that warming enhanced destabilization and breakdown of aggregates (Poeplau et al., 2017, 2020). It may be that physical responses to warming are mediated by different mechanisms across diverse ecosystems, such as aggregate binding agents, SOM quality, and soil texture (Costa et al., 2018; Lavee et al., 1996; Poeplau et al., 2020; Tisdall and Oades, 1982).

4.2. Microbial temperature responses are attenuated by long-term warming

CUE and respiration were less temperature sensitive in macroaggregates isolated from warmed compared to control soils, possibly associated with decreased substrate availability and adjustments of microbial physiology and activity, shifts in community structure and function (DeAngelis et al., 2015; Pold et al., 2016), or thermal adaption of microbial communities with long-term warming (Allison, 2014; Frey et al., 2013; Melillo et al., 2017). Another explanation for the decrease of temperature sensitivity might be that warming-associated soil drying enhanced substrate limitation and reduced microbial activity (Luo et al., 2001). However, we found little evidence for microbial thermal responses in microaggregates, likely attributed to greater physical protection of SOM that reduced microbial accessibility to substrates and microbial temperature responses to long-term warming.

CUE and respiration were less temperature sensitive in microaggregates than in macroaggregates derived from control soils (Table 2), possibly due to greater physical protection (Fig. S3, Table S1) that hampered the adsorption and desorption processes of substrates available to microbes (Conant et al., 2011). Microaggregates also had greater DOC concentration that should reduce the microbial requirement for enzyme production. The lower temperature sensitivity in microaggregates might also be associated with more diverse ecological niches (Bach et al., 2018; Davinic et al., 2012; Ivanova et al., 2015) and more abundant oligotrophic microbes (Gupta and Germida, 2015; Trivedi et al., 2017).

4.3. Limitation of long-term warming effects as a self-reinforcing feedback to climate

Because CUE is a metric linking microbial growth to soil C loss (Allison, 2014; Tucker et al., 2013), the reduced growth and respiration with long-term warming suggest a limitation to the microbial self-reinforcing feedback of soil C loss to the atmosphere. Chronic warming at this site has induced a nonlinear pattern of soil C loss, with phases of soil C decay punctuated by changes in the microbial community structure (Melillo et al., 2017). We sampled during the most recent phase, characterized by similar CO_2 fluxes from the warmed and control plots. Microbial respiration contributes more than 80% of total belowground respiration (Melillo et al., 2002), with warmed soils showing persistently suppressed microbial biomass, activity and substrate availability (Frey et al., 2008; Melillo et al., 2017; Pold et al., 2017). This is consistent with a more generally observed trend that variables such as thermal acclimation, nutrient limitation, and altered microbial accessibility to labile SOM are likely to attenuate the impacts of warming on soil C loss to the atmosphere (Romero-Olivares et al., 2017).

Long-term warming tended to increase microbial turnover time in microaggregates but not in macroaggregates. Warming effects that could explain the longer turnover time in microaggregates may include lower activity of predators and grazers (Kaiser et al., 2014) that reduced cell death (Li et al., 2019), or selected microbial groups with slower growth and turnover rates (Bernard et al., 2007; Eilers et al., 2010; Liu et al., 2020; Morrissey et al., 2019). Thus, the greater energetic constrains under warming, namely lower microbial organic C uptake (Fig. S4), in microaggregates was compensated by a longer turnover time of microbial biomass (Spohn et al., 2016). Longer turnover time could also be caused by an increasing fraction of dormant cells with long-term warming (De Nobili et al., 2001). Although longer turnover time might reduce necromass formation and so contribute to depleted SOM pools (Creamer et al., 2019; Hagerty et al., 2014), it can also reduce the degradation of SOM and soil C loss (Spohn et al., 2016). Our findings suggest that microbial turnover was mediated by soil physical protection (*i.e.*, aggregate size) and associated changes in substrate availability and microbial communities.

4.4. Different environmental drivers of microbial efficiency and turnover

Microbial CUE and turnover time were driven by different environmental factors, suggesting that microbes have different metabolic allocation strategies (Hagerty et al., 2018) and thermal responses over long-term warming (Tucker et al., 2013; Wagai et al., 2013). Incubation temperature was the main driver of CUE, due to fast increase of respiration with little change in growth during the short-term incubation. However, the lack of long-term warming as the main driver for CUE was caused by a decrease of both respiration and growth in the long-term warmed soils. Aggregate size was the main driver of turnover time, possibly due to aggregate-driven differences in microbial communities (Davinic et al., 2012; Mummey et al., 2006), functional groups (Bernard et al., 2007; Morrissey et al., 2019) and predation pressure and mortality (Kaiser et al., 2014; Li et al., 2019) that were mediated by substrate availability (Figs. 1 and S1) and physical protection (Fig. S3 and Table S1).

Temperature sensitivity of CUE and turnover time were also differently driven by environmental drivers. The CUE temperature sensitivity was driven by long-term warming and aggregate size, while temperature sensitivity of turnover time was driven mostly by aggregate size, suggesting the critical role of physical protection (i.e., greater fractions of silt/clay and MAOM in microaggregates; Table S1 and Fig. S3) (Poeplau et al., 2020) in mediating microbial adaptation over long-term climate warming.

4.5. Potential aggregate isolation and storage effects on microbial parameters

Compared to the bulk soil, research on finer resolution of soil matrix can provide more mechanistic insights in microbial activities and microbial communities that utilize different SOM pools (Bailey et al., 2013; Poeplau et al., 2020; Six et al., 2000; Totsche et al., 2018; Waring et al., 2020) over long-term warming. We chose to dry the fresh soil at low temperature (4 °C) to a optimal moisture (~10%) before aggregate isolation (dry sieving) to reduce disturbance effects on microbial parameters and to facilitate the aggregate separation process (Bach and Hofmockel, 2014). This optimal moisture dry sieving method has more advantages than the wet sieving, which has more dramatic wet-dry cycles that would significantly interfere with microbial activities and enzyme production (Allison and Jastrow, 2006; Bach and Hofmockel, 2014; Dorodnikov et al., 2009). Because all the warming and aggregate treatments had the experienced the same incubation conditions, the treatments effects on microbial parameters should be valid (Peng et al., 2017; Six and Paustian, 2014; Tian et al., 2015).

Storage duration for soil aggregates and freeze-thaw process might affect microbial enzyme activities. Our aggregates samples were isolated and frozen at −20 °C, stored for about eight months, and weighed immediately before the enzyme assays were conducted at the room temperature. Freezing soil samples might affect enzyme activities compared to fresh soil samples, but the changes were tolerable if the storage has the same proportional effect on all treatments (DeForest, 2009; Peoples and Koide, 2012). Because most soil enzymes are extracellular (Kandeler, 1990; Schimel et al., 2007), our study reflects the practice of other studies in assuming that the loss of enzyme activity due to freezing should be small and consistent across treatments (Finley et al., 2018; Liu et al., 2020; Peoples and Koide, 2012).

5. Conclusions

Nearly three decades of warming caused decreases in microbial growth and respiration, and has reduced substrate availability to microbes and enzyme activity, especially in macroaggregates. In contrast, there was little change in microbial CUE and biomass turnover time. We found that CUE and respiration became less temperature sensitive in the macroaggregates from warmed compared to control plots, suggesting that microbial thermal responses co-vary with individual SOC stabilization processes. Our findings suggest that SOM is more vulnerable to microbial degradation in less physically protected soil compartments (i.e., macroaggregates), where microbes are more temperature sensitive over long-term warming.

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.

Acknowledgements

The authors want to thank W. Werner for soil sampling; M. Spohn, S. J. Blazewicz, P. Dijkstra, C.M. Kallenbach and K. Papp for technical discussions; M. Arredondo, H. Mashayekhi and H.P. Shang for sample analyses. The work was funded by the DOE Terrestrial Ecosystem Science Program (DE-SC0016571) and the NSF Long Term Ecological Research Network (LTER) (DEB: 1832210).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.108055.

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