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LETTER

Elevated CO_2 and warming cause interactive effects on soil carbon and shifts in carbon use by bacteria

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Abstract

Accurate predictions of soil C feedbacks to climate change depend on an improved understanding of responses of soil C pools and C use by soil microbial groups. We assessed soil and microbial C in a 7-year manipulation of CO_2 and warming in a semi-arid grassland. Continuous field isotopic labelling under elevated CO_2 further allowed us to study the dynamics of the existing C (Old C) in soil and microbes as affected by warming. Warming reduced soil C under elevated CO_2 but had no impact under ambient CO_2. Loss of soil C under warming and elevated CO_2 was attributed to increased proportional loss of Old C. Warming also reduced the proportion of Old C in microbes, specifically the bacteria, but not the fungi. These findings highlight that warming impacts are C pool and microbial taxa dependent and demonstrate interactive effects of warming and atmospheric CO_2 on soil C.

Keywords

Bacteria, carbon, climate change, elevated CO_2, microbial C use, microbial communities, microbial function, soil, warming.

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INTRODUCTION

The global soil carbon (C) stock is about three times larger than that in the atmosphere, so changes in its size have the potential to enhance or counteract ongoing climate changes via the control of atmospheric CO_2. Future climate will include both elevated atmospheric CO_2 (eCO_2) and warmer conditions, each of which is expected to impact terrestrial C cycling. Despite increases in plant C inputs with eCO_2 (Lu *et al*. 2013; Yue *et al*. 2017), empirical and synthesis efforts point at small effects or even losses of soil C with eCO_2 across multiple ecosystems (Norby & Zak 2011; Sillen & Dieleman 2012; Procter *et al*. 2015; Black *et al*. 2017; Yue *et al*. 2017). These variable responses appear to be associated with variation in C turnover rates (van Groenigen *et al*. 2014, 2015) across different C pools (van Groenigen *et al*. 2017) for which the mechanisms are not clear. The observed effects of warming on soil C pools are likewise highly variable and the main driving factors remain uncertain (van Gestel *et al*. 2018). Increased decomposition commonly occurs with warming but its impact on C stocks may be offset by increases in productivity (Lu *et al*. 2013; Yue *et al*. 2017). However, mechanistic understanding of the drivers of this balance is still limited (Conant *et al*. 2011), given the multiplicity of factors at play. Recent studies suggest a role of differential responses of soil organic matter pools (Hopkins *et al*. 2012; Frey *et al*. 2013; Pries *et al*. 2017) and of microbial abundance, community composition and C use (Luo *et al*. 2014; Streit *et al*. 2014; Chen *et al*. 2015; Garcia-Palacios *et al*. 2015) as well as a role of warming in the stabilisation of microbial C (Liang & Balser 2012). Given the complexity of the individual impacts of eCO_2 and warming and the paucity of long-term multifactorial experiments, there is substantial uncertainty about how these two factors combined will impact soil C stocks and the associated feedbacks to climate change (Pendall *et al*. 2004; Crowther *et al*. 2016). Targeting the main sources of this uncertainty should improve our ability to predict future C balance (Bradford *et al*. 2016).

One main source of current uncertainty stems from the difficulty in assessing the actual standing stocks of C and their responses to current or simulated climate changes (Köchy *et al*. 2015). High spatial heterogeneity, high background levels, soil sampling strategies, post-sampling handling and short experimental duration (Gifford & Roderick 2003; Lehmann *et al*. 2008; Hungate *et al*. 2009; Hoffmann *et al*. 2017) all hinder accurate assessment and thus, detection of changes over time. Furthermore, different C pools are likely to respond differently to environmental changes (Carrillo *et al*. 2011; Groenigen *et al*. 2017) potentially leading to net effects that mask changes within specific pools. Partly due to these combined challenges, most efforts to assess soil C responses have focused solely on C decomposition (e.g. Dieleman *et al*. 2012; Zhou *et al*. 2016), while soil C formation, the other driver of C stocks, is less frequently considered. The paucity of mid- to long-term, direct assessments of total soil C stocks and their component pools in response to climate factors is limiting our ability to evaluate soil C feedbacks to climate change (Bradford *et al*. 2016; Crowther *et al*. 2016). Labelling of plant biomass with C isotopic tracers in intact ecosystems, particularly if done for an extended time, offers multiple

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opportunities to improve our ability to assess shifts in soil C pools. Isotope tracers allow us to follow the dynamics of different soil C pools including the net accumulation of newly fixed C and the net loss of native C.

Our limited understanding of the role of soil microbes in both C decomposition and formation under environmental change is another critical source of uncertainty about potential soil C feedbacks to climate change (Bradford *et al.* 2016). Most research on the response of microbes has focused on their role as decomposers, mainly from short-term studies on soil respiration (e.g. Eliasson *et al.* 2005; Zhou *et al.* 2016). This understanding underlies the current representation of microbes in Earth System models (Todd-Brown *et al.* 2012). However, a more refined understanding and representation of microbial responses and functions is necessary for more realistic projections (Wieder *et al.* 2015; Luo *et al.* 2016), but advances are still hindered by limited empirical and field-based knowledge. A number of recent advances highlight areas for which greater understanding is needed. These include: (1) different microbial groups are not likely to respond in similar ways to climate changes (Castro *et al.* 2010; Andresen *et al.* 2014; Luo *et al.* 2014), (2) initial responses may dissipate or shift over time (Blankinship *et al.* 2011; Craine *et al.* 2013), (3) microbial groups differ in the pools of C they use and these substrate preferences may shift under a new environment (Andresen *et al.* 2014; Streit *et al.* 2014), and (4) their contribution to soil C decomposition and formation is likely to differ due to contrasting physiology and efficiency in their use of C (Liang & Balser 2012; Frey *et al.* 2013; Allison 2014). It is clear that refining our understanding of how microbial groups use C sources under future climates will spur progress in understanding future C cycling. Long-term, multifactorial, field simulations of future conditions incorporating long-term C isotopic labelling allow for assessing microbial use of C pools over time. Combining these with the concomitant observation of soil C pool dynamics will provide empirical evidence of the linkages between microbial C use, microbial community composition and soil C stocks.

Our first aim was to study the dynamics of soil C over time under warming, eCO_2 and their combination at the Prairie Heating and CO_2 Enrichment (PHACE) experiment. PHACE was a 7-year climate change experiment that combined Free Air CO_2 Enrichment (FACE) and ecosystem warming in a native semi-arid mixed grass prairie in Wyoming, USA. The continuous fumigation with isotopically labelled CO_2 in the eCO_2 treatments allowed us to track the dynamics of the pools of unlabelled, pre-existing soil C (Old C) and New C since the start of the experiment both under unwarmed and warmed conditions. Thus, our second aim was to examine the impact of warming on the Old C pool and its use by microbes. For this, we assessed microbial community composition and the use of Old C by the microbial biomass over time and by specific microbial functional groups. Furthermore, we investigated the role of potential drivers of the shifts in microbial C use. Parton *et al.* (2007) used the Daycent model to simulate the ecosystem-level responses of PHACE over 10 years and Mueller *et al.* (2016) studied the responses of plant biomass over time. Here, we hypothesised that warming would lead to an increase in soil C due to the suppression of decomposition with drying from warming and no strong shift in plant inputs (Mueller *et al.* 2016; Parton *et al.* 2007; Fig. 1a). We hypothesised that in this water-limited system, eCO_2 would decrease soil C due to higher decomposition driven by higher soil moisture, combined with no strong impacts on belowground biomass inputs (Mueller *et al.* 2016; Parton *et al.* 2007; Fig. 1b). Thus, we hypothesised that, when combined, the effects of eCO_2 and warming on decomposition would offset one another (Parton *et al.* 2007) but soil C would increase due to positive, interactive impacts of the combined factors on plant biomass (Mueller *et al.* 2016; Fig. 1c).

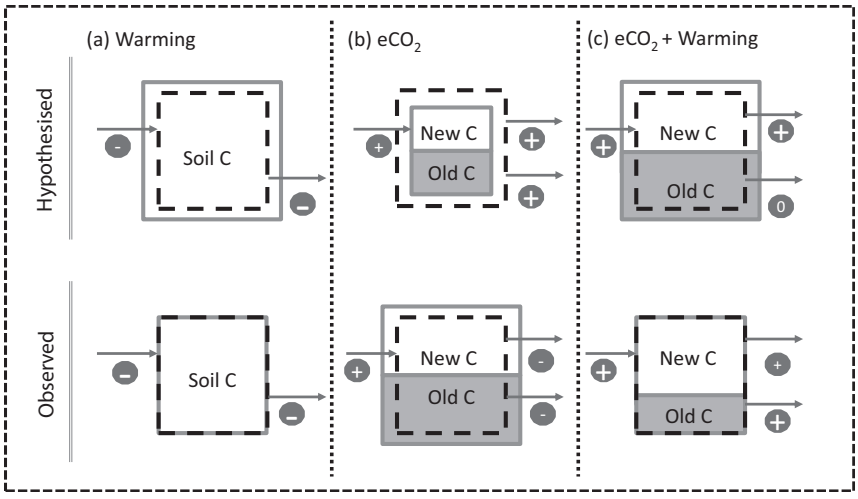

Figure 1 Hypothesised and observed relative responses of soil C and fractions of New and Old C to Warming, elevated CO_2 and combined factors at the PHACE Prairie Heating and CO_2 Enrichment experiment native mixed-grass prairie. The dashed box represents the size of the soil C pool under ambient, untreated conditions and solid line boxes represent the relative change. New C and Old C pools shown for eCO_2 treatments, where isotopic-based separation was possible. Proportion of New C and Old C is 0.5 for simplicity (See Fig. 4 for actual values) and relative change with warming is depicted. Arrows going in represent C inputs and arrows going out represent C losses. Pluses/minuses indicate increase/decrease in inputs/losses relative to control conditions. "0" indicates no responses. Relative sizes of pluses and minuses represent relative magnitude of change in input and losses.

We hypothesised that warming would not change the proportion of Old C in soil as the increase in New C would be counteracted with greater relative microbial decomposition of this pool (Fig. 1b and c).

METHODS

Experimental site and manipulation

The PHACE experimental site is located at the USDA-ARS High Plains Grassland Research Station (1930 m.a.s.l.), 15 km west of Cheyenne, WY, USA (41 ° 11′ N, 104° 54′ W). The ecosystem is a northern mixed grass prairie dominated by the perennial C4 grass *Bouteloua gracilis* (H.B.K) Lag. and two C3 grasses, *Hesperostipa comata* Trin and Rupr. and *Pascopyrum smithii* (Rydb.). Mean annual precipitation is 384 mm; mean air temperatures are 17.5 °C in summer and −2.5 °C in winter (NOAA, 1994). Soils are Mollisols (fine-loamy, mesic Aridic Argiustoll, mixed Ascalon and Altvan series) with 0.96 kg C m^{-2} (0–5 cm). The PHACE experiment was a factorial combination of CO_2 and warming with five replications. CO_2 was raised during the growing season to 600 ± 40 ppmv via Free Air CO_2 Enrichment installed in 3.4-m-diameter rings. Increased canopy temperature (1.5 °C daytime/3 °C night, year round) was generated with a hexagonal array of 1000-watt Mor FTE infrared heaters (Comstock Park, MI, USA) attached to a 1.5-m-high frame (Kimball *et al*. 2008). The CO_2 treatment began in early April 2006 and heating in early April 2007. Soil volumetric water was monitored continuously using frequency domain reflectrometry and nitrogen availability was assessed with buried ion exchange resins (Mueller *et al*. 2016).

Soil sampling, processing and analysis

Soils were sampled once a year in late July starting the year before treatments began until the experiment ended (2005–2013, with the exception of 2006) by collecting and compositing four 3-cm diameter, 15-cm deep cores from each plot which were split among 0–5 and 5–15-cm depths. A subsample of the composited soil was sieved to 2 mm and further inspected to remove any visible particulate organic matter. Following this, subsamples were treated to remove carbonates by shaking with 1 M H_3PO_4 (Sherrod *et al*. 2002) and then filtered, rinsed and dried to remove the acid. After grinding to powder, soils were weighed for C and $\delta^{13}C$ analysis on a Finnigan DeltaPlus XP connected to a Carlo Erba NC-2500 elemental analyser via a Finnigan ConFlo III open-split interface.

Microbial C and ^{13}C and microbial community PLFA and ^{13}C-PLFA

Within 24 h of collection, 2 mm sieved subsamples were processed for microbial C and $\delta^{13}C$. Microbial C was assessed with chloroform fumigation extraction (CFE, 0.05 M K_2SO_4). Extracts were analysed for total C on a Total Organic Carbon analyser (Shimadzu TOC-VCPN; Shimadzu Scientific Instruments, Wood Dale, IL, USA) after treating for carbonates (1 M H_3PO_4 at 1 μL per 10 mL of extract). The $\delta^{13}C$ of extracts was measured on a dried aliquot (60 °C) using a Finnigan DeltaPlus XP connected to a Carlo Erba NC-2500 elemental analyser via a Finnigan ConFlo III open-split interface. Microbial $\delta^{13}C$ was calculated as $\delta^{13}C$ mic = (δ^{13}Cf *Cf – δ^{13}C uf *Cuf)/(Cf – Cuf), where δ^{13}Cf and δ^{13}Cuf values are isotopic ratios (per mil) and Cf and Cuf are the C content of the extracts of fumigated and unfumigated soils respectively.

Phospholipid fatty acid composition (PLFA) and $\delta^{13}C$-PLFA were determined on soils from the last sampling date, in 2013. A 6-g subsample of soil that had been frozen fresh and freeze-dried was extracted with methanol–chloroform–phosphate buffer (2:1:0.8 in volume) and fractionated (Zelles and Bai 1993) with a silica gel column, mild alkaline methanolysis was used to produce phospholipid fatty acid methyl esters which were then purified with NH_2 aminopropyl columns. PLFAs were analysed on an Agilent 6890N chromatograph (Agilent Technologies, Wilmintong, Delaware, USA). Peak identification was done with the Sherlock Microbial Identification System (MIDI, Inc., Newark, NJ, USA). Individual PLFAs were quantified in relation to an internal standard of known concentration (20:0 ethyl ester). For $\delta^{13}C$ of individual PLFAs, extracts were run on a Trace GC Ultra gas chromatograph (Thermo Electron Corp., Milan, Italy) coupled with a Delta V Advantage isotope ratio mass spectrometer through a GC/C-III. The $\delta^{13}C$ values were corrected for the contribution of methanol C during sterification using mass balance for each fatty acid.

Estimation of the fraction of Old C in soil, microbial and PLFA C

To investigate impacts of warming on C dynamics and microbial use, we take advantage of the continuous addition of ^{13}C-depleted CO_2 in the eCO_2 plots to estimate the proportion of Old soil C in soil, microbial biomass and PLFAs through isotopic partitioning using measured isotopic values (See Appendix S1). Old C is defined as the C existing prior to CO_2 enrichment, including any plant C. The proportion of Old soil C-derived C in soil and microbial biomass was calculated as:

$$\text{Fraction Old C}_{(\text{Soil,Microbial Biomass})} = \frac{(\delta^{13}C_{\text{Soil,Microbial biomass}} - \delta^{13}C_{\text{soil aCO2}})}{(\delta^{13}C_{AG} - \delta^{13}C_{\text{soil aCO2}})}$$

where $\delta^{13}C_{\text{Soil, Microbial biomass}}$ are the $\delta^{13}C$ of those pools at each time and for each eCO_2 plot, $\delta^{13}C_{\text{Soil aCO2}}$ is the $\delta^{13}C$ of the soil C in the ambient CO_2 plots in each year. There was no influence of warming on the $\delta^{13}C$ soil in either CO_2 treatment (Table S1), thus no bias was introduced. $\delta^{13}C_{AG}$ is the $\delta^{13}C$ of the aboveground plant biomass in each of the eCO_2 plots and in each year from the annual biomass sampling (Mueller *et al*. 2016); thus accounting for shifts in C3–C4 abundance in the eCO_2 plots. Aboveground rather than root biomass $\delta^{13}C$ was used as the latter includes pre- and post-enrichment C (Carrillo *et al*. 2014a). For PLFA, the fraction of Old C was estimated as in Hopkins *et al*. (2014) following Kramer & Gleixner (2006) so that

$$\text{Fraction Old } C_{PLFAi} = \frac{(\delta^{13}C_{PLFAi-eCO2} - \delta^{13}C_{PLFAi-aCO2})}{(\delta^{13}C_{AG\ eCO2} - \delta^{13}C_{AG\ aCO2})}$$

where PLFAi-eCO_2 is an individual PLFA in each of the eCO_2 plots, PLFAi-aCO_2 is an individual PLFA averaged across ambient CO_2 plots, AG-eCO_2/aCO_2 is the above-ground biomass as described above. FACE-derived C in all pools is defined as the remaining fraction after subtracting the fraction Old C.

Quantification of soil respiration and C priming

The day after the last soil collection (2013), incubations of soil with and without ^{13}C-labelled glucose additions were set up to assess whether treatments had modified the susceptibility of soil C to decomposition in response to C addition (priming). Twenty grams of field-moisture (14% average moisture content) soil was incubated in sealed 500-ml jars for 44 h at room temperature (see Carrillo *et al.* (2011) for details). One set of soils received 2 ml of H_2O and the other was amended with 2 ml of a solution containing 250 mg of 6.96 atom% ^{13}C glucose and 38.3 mg NH_4Cl. The addition aimed to represent approximately 35% of the microbial biomass C and to contain a C:N of 10:1 to prevent N limitation. The $\delta^{13}C$ of CO_2 released from both sets was used to calculate the fraction of the respiration derived from soil C in the glucose-amended soils as

$$\text{Fraction Soil } C_{CO2} = \frac{(\delta^{13}C_{CO2\ amended\ soil} - \delta^{13}C_{glucose})}{(\delta^{13}C_{CO2\ unamended\ soil} - \delta^{13}C_{glucose})}$$

These fractions were applied to respiration rates and priming was calculated as the difference between soil-derived CO_2 in amended soils and CO_2-C produced from unamended soils.

Statistical analyses

We evaluated treatment effects on soil %C and microbial C with repeated measures ANOVA with CO_2, warming and their interaction as factors. Both variables showed only effects of time and in the case of soil C some pre-experimental (2005) differences across plots (See Appendix S2). To account for these, we then focused on evaluating the change in soil % C (ΔC, defined as the difference between the %C value of each plot in each year and its pre-treatment value in 2005). These models included CO_2, warming, year (discrete, accounting for variation over time without assuming a linear trend) and their interactions as fixed effects and plot (as a random effect to specify that values within a plot across years were repeated measures) as factors. This approach accounted for error associated with site spatial variation, sampling and processing across time so that treatment effects on change relative to pre-experimental conditions could be assessed. Priming was analysed with two-factor (CO_2 and warming) ANOVA. Nonlinear regression was used to fit the decrease in the fraction of Old C over time. Effects of warming on the parameters of best regression fits and on the fractions measured in particular PLFA functional groups were evaluated with t-tests. Effects of treatments on PLFA profiles were analysed with principal component analysis (PCA) on correlations and two-factor ANOVA on PCs.

To investigate potential drivers of shifts in microbial C use, we explored the role of root biomass, water availability and nutrients in plants and soil as predictors of microbial Old C use with multiple regression and model selection. All possible multiple regression models of microbial C use (total biomass and microbial groups (Table 1)) as a function of these four variables, were compared and the best models based on corrected Akaike Information Criterion (AICc) within 2 AICc units were selected. For this analysis we used values of potential predictors averaged across years in each plot over the course of the experiment. These observations and their corresponding methodologies have been presented previously (Mueller *et al.* 2016). Root biomass (0–15 cm) was used as an indicator of the amount of plant input. Root % N was used as a proxy of plant N concentration. Water content was volumetric water content from continuous soil moisture

Table 1 Best multiple regression models of variables describing the microbial use of Old C (as fraction of Old C) at PHACE experiment against potential predictors. Old C in microbial biomass based on chloroform fumigation extractions. Old C in PLFA based on grouping of PLFAs into functional groups. Estimation of fraction of Old C based on C isotopic partitioning. Best models within two AICc units (up to three) presented including direction, value and *P* value of significant estimates. NS: not significant model/predictor ($P > 0.10$). See Methods for details on the evaluated predictors. Potential predictor variables were averages across years

						Predictor					
						Soil moisture		Root biomass		Root % *N*	Soil avail *N*
	Variable	Model	AICc	R2	*P*	Estimate	*P*	Estimate	*P*		
Old C microbial biomass	Final fraction	1	−25.04	0.67	0.02	0.0023	0.03	−7.94E-05	0.03	NS	NS
		2	−23.54	0.30	0.10	0.0021	0.10	NS		NS	NS
		3	−23.48	0.30	0.10	–	NS	−7.08E-05	0.10	NS	NS
	1/rate of decrease	1	14.51	0.30	0.10	–	NS	0.0005	0.10	NS	NS
Old C	General markers	1	−6.52	0.28	0.12	0.0046	0.12	–	NS	NS	NS
PLFA microbial functional groups	Gram-positive bacteria	1	−7.94	0.40	0.05	0.0055	0.05	–	NS	NS	NS
	Gram-negative bacteria	1	−9.82	0.33	0.10	0.0036	0.10	–	NS	NS	NS
	Actinobact	1	−3.77	0.38	0.08	0.0056	0.08	–	NS	NS	NS
	Fungi	1	NS	NS	NS	–	NS	–	NS	NS	NS

measurements down to 15 cm. Available N in soil was based on accumulation in ion exchange resins. All variables were checked for normality and homoscedasticity and log transformed when needed. Analyses were performed with JMP (version 11.0; SAS Institute, Cary, NC, USA).

RESULTS

Total soil C

Warming and eCO_2 interacted to significantly affect ΔC (difference between %C in each year and pre-experimental values) at 0–5 cm (Fig. 2). In shallow soils, ΔC increased with eCO_2 but only at ambient temperature (CO_2 effect at ambient temperature: $P = 0.033$, $F = 6.64$ D.F. =1). In contrast, soil ΔC decreased with warming, but only in combination with eCO_2 (Warming effect at eCO_2: $P = 0.05$, $F = 5.31$, D.F = 1). Overall values of ΔC fluctuated across years (significant effect of year but independent of treatment), which is attributable to error associated with spatial variation and processing and analyses of samples (Fig. 2). Soil C % and microbial C values varied over time but did not show treatment effects (See Appendix S2).

Soil respiration and priming

Soil respiration measured in the soil incubations without glucose addition was significantly higher under eCO_2 (Fig. 3a). This effect was mainly due to a greater amount of available substrate, as suggested by a much weaker CO_2 effect on the respiration rate per unit of soil C (Fig. 3b). Despite increased respiration with eCO_2, the priming assessment revealed that the stimulation of soil C decomposition caused by C addition was reduced under eCO_2 (Fig. 3c).

Old C in soil and microbial biomass over time

The fraction of Old soil C (derived from the C existing at the time the field CO_2 addition started), decreased significantly over time to less than 0.88 at the end of the experiment (Fig. 4a). The decline in the fraction of Old soil C best fits a two-pool exponential decay model indicating a faster decline in the early stage (Fig. 4a). This decay dynamic reflects the decline over time in the available C that was present in the original soil organic matter. The initial rate of decline was faster (decay constant = 2.01 under Warming vs 0.97 under Ambient) and the overall decrease reached lower values under warming (Fig. 4a), but these parameters were not statistically different. The fraction of Old soil C in the microbial biomass (with CFE) decreased faster than that in soil and reached about 0.6 by the end of the experiment (Fig. 4b). The decline of the fraction of Old C in the microbial biomass best fit a single-pool, three-parameter exponential decay model approaching a stable value. This value indicated that the microbial biomass contained a significantly lower fraction of old C under warming ($p = 0.024$, t-test, n = 5), thus showing reduced proportional use of Old soil C (thus increased use of New, FACE-derived C) with warming. The fraction of Old C in microbial biomass and soil were positively related (Fig. 4c).

Microbial communities and use of Old C

Elevated CO_2 was the main driver of the microbial community structure (Fig. S3a); however, warming impacted particular groups (Fig. S3b). Elevated CO_2 decreased the relative abundance of actinobacteria while it increased the abundance of fungi, as well as the ratio of fungi to bacteria and protozoa (Fig S3b). Warming increased the Gram-positive bacteria but reduced protozoa. The partitioning of individual PLFA C into Old soil C and FACE-derived C, followed by grouping into functional groups indicated that at the end of the experiment the overall microbial C was close to 50% Old C-derived (general microbial markers, that is, markers of all microbes, Fig. 5a). The use of Old C was not uniform across functional groups as the fraction of Old C in the bacterial markers was more than double than that in the fungal markers. While we did not detect strong shifts in microbial community structure with warming, isotopic partitioning demonstrated clear changes in microbial use of C under warming. The fraction of Old C in general markers significantly decreased with warming (Fig. 5a), in accordance with the independent estimates based on CFE microbial biomass (Fig. 4b). The overall decrease in the proportional use of Old C with warming was also consistent with a significant decrease in the fraction of Old C with warming in the bacterial markers, which did not occur for the fungal markers (Fig. 5a). Together, C tracing in microbial biomass and PLFA indicated reduced use of Old C, and thus greater use of FACE-derived C under warming conditions, driven by bacteria. As with the CFE microbial biomass, the fraction of Old C in the general and bacterial markers, but not fungal markers, was positively related to the fraction of Old C in soil (Fig. 5b).

Root biomass and particularly soil moisture were significant predictors of microbial use of Old C (Table 1). Soil moisture was positively related to the fraction of Old C in CFE microbial biomass and general microbial PLFA markers as well as bacterial markers. Root biomass, on the other hand, was negatively related to the CFE fraction of Old C.

DISCUSSION

Our multifaceted, 8-year study of soil C in a native mixed-grass prairie contradicted our hypotheses that soil C would increase with warming, decrease with eCO_2 and increase under combined warming and eCO_2. Moreover, it revealed interactive effects of CO_2 and warming previously not predicted by experiment-specific ecosystem response modelling (Parton *et al*. 2007). These findings demonstrate the necessity of long-term, multifactor global change manipulations to improve predictive understanding. The long-term C labelling and tracking revealed that warming had contrasting effects in the Old and New pools of C, also contradicting our hypothesis. Our microbial analysis suggested potential linkages between these effects of warming and shifts in C use particularly by the bacterial members of the microbial community.

Despite higher soil moisture with eCO_2 and lower with warming (Mueller *et al*. 2016), our observations of increased soil C with eCO_2 and no clear effect of warming alone are in contrast to our predictions of decreased soil C with eCO_2 due

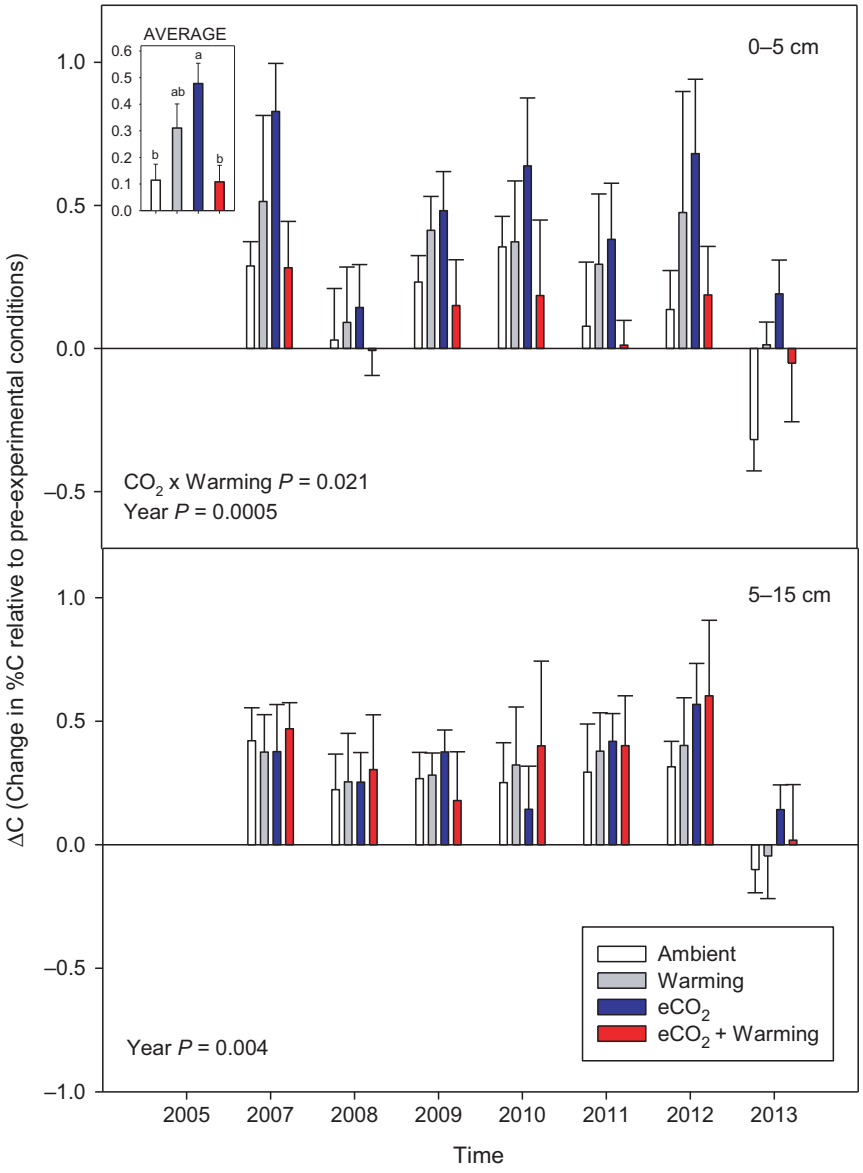

Figure 2 Change in C percentage in soil relative to pre-experimental conditions (ΔC) over time at the PHACE experiment. Values are % soil C in each plot each year minus its value in 2005. Values are averages with standard error (n = 5). Average values across years shown in insert; different letters indicate significant $P < 0.05$ (Student t-test).

to greater decomposition with more water availability and increased soil C with warming due to suppressed decomposition due to drying (Fig. 1b). Elevated CO_2 may have increased soil C via greater belowground C inputs (Carrillo *et al.* 2014a; Mueller *et al.* 2016). In addition, our data suggest that lower susceptibility to C loss under eCO_2 may have also played a role. After eight growing seasons of eCO_2, soil C was less sensitive to priming induced by labile C addition (Fig. 3), which is consistent with observations of decreased decomposition of soil C under eCO_2 in controlled-environment studies using PHACE species and soil (Carrillo *et al.* 2014b, 2016). While increases in C turnover with eCO_2 in soil pools cannot be ruled out and are likely to have occurred concomitantly (van Groenigen *et al.* 2014; Groenigen *et al.* 2017), they appear to have been offset by greater plant inputs and decreased overall susceptibility to decomposition, leading to significantly greater soil C storage with eCO_2 (Fig. 1). In the case of warming, the lack of a significant increase in soil C, as was predicted, could have been due to lower belowground inputs (Mueller *et al.* 2016) offsetting the decreased decomposition with soil drying (Fig. 1). Our findings, thus, suggest that despite the often important role of water limitation for decomposition in this system, further mechanisms such as changes in the susceptibility of organic matter to decay, need to be invoked to explain climate change impacts, particularly those of CO_2.

Our results provide evidence that the effect of warming was dependent on CO_2 conditions: soil C was lost with warming under eCO_2, but not under ambient CO_2. This C loss was sufficient to offset the positive impact of eCO_2 alone and

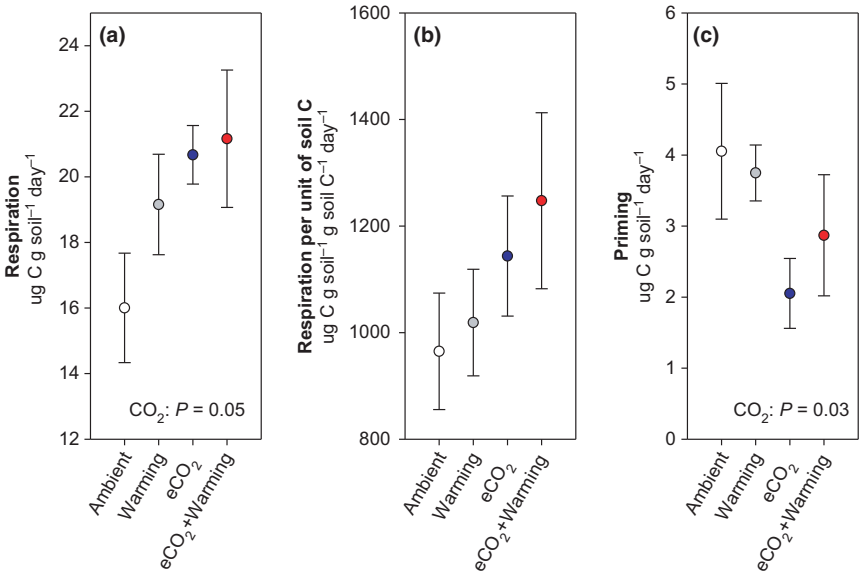

Figure 3 Soil C respiration and soil priming in incubated PHACE soil after 7 years of experimental manipulation. Respiration per gram of soil in unamended soils (a) and standardised per gram of soil carbon (b) and response of respiration to addition of C, or priming (c). Values are averages for 0–5 cm soil with standard error (n = 5). Note different scales and units of y-axes. P values from two-factor ANOVA with interactions.

moreover, it was sufficient to offset the increase in belowground input with warming that occurred under eCO_2 (Mueller *et al.* 2016) (Fig. 1). Reduction in soil C with warming and interactive effects with CO_2 are in contrast to Parton *et al.*'s (2007) predictions of suppression of decomposition with reduced moisture independent of CO_2. Increased soil moisture under eCO_2 (Morgan *et al.* 2011; Mueller *et al.* 2016) could have removed water limitation to decomposition, thus enhancing decomposition with warming (Pendall *et al.* 2013). However, isotopically distinguishing Old soil C across time indicated that the proportion of Old C declined with warming. Our findings imply that a greater decrease in the fraction of Old C with warming, particularly early after treatment initiation, contributed to its offsetting of the positive effects of eCO_2 on total soil C. This is consistent with reports of increased susceptibility to warming of C pools that vary in age from years to decades (Hopkins *et al.* 2012; Pries *et al.* 2017). Our results demonstrate that at least for this grassland the impacts of warming are dependent on CO_2 conditions and call for caution when extending observations of warming under ambient CO_2 to future conditions with higher atmospheric CO_2. Our findings further suggest that considering differential responses of soil C pools may improve our ability to predict interactive impacts of climate factors on C dynamics.

The joint assessment of Old soil C in soil organic matter and in microbes over time revealed relationships between microbial C use and soil C pools. The increased loss of C (total and fraction of Old C) with warming (compared to unwarmed eCO_2) occurred in parallel with a reduction in the Old C in the total microbial biomass that was sustained over the experimental period. This reduction, in turn, could be explained by the reduced use of Old C (and conversely, increased use of plant C including dead roots, exudation and other rhizodeposits) by the bacteria. Hence, our observations indicate that the impact of warming did not generally occur for all microbial C use, but that the bacteria were more directly affected. Decreased incorporation of Old C into microbial biomass, and the bacteria in particular, could be interpreted as reduced use of this pool, which in turn, could be associated with less decomposition of this substrate. This would result in an inverse relationship between the proportion of a particular pool in soil and that in microbes. In contrast, we observed clear positive relationships between Old C in soil and in microbial biomass (Fig. 4c) as well in as the independently measured PLFA markers (Fig. 5b). This relationship could be explained by the availability of C to microbes: as less Old C is present, less is available to be incorporated into microbes. In turn, less Old C-derived bacterial biomass can contribute to soil organic matter formation, further contributing to the positive relationship between Old C in soil and microbial/bacterial Old C (Bradford *et al.* 2013).

Our exploration of other driving variables suggested that other factors, besides overall Old C availability, could have further contributed to the reduction in the fraction of Old C in the bacteria. Lower use of Old soil C by bacteria with the warming treatment was related to greater root biomass. This inverse relationship is expected and consistent with a shorter field study in an alpine system showing increase in microbial use of Old C with warming, which was linked to reduced plant inputs (Streit *et al.* 2014). However, we determined that an even stronger driver of reduced Old C use was the decrease in soil water, which occurs with warming at PHACE both under aCO_2 and eCO_2 (Morgan *et al.* 2011; Mueller *et al.* 2016a). Potential explanations for this include reduced diffusion of Old C to bacteria with lower watered-filled pore space (Manzoni *et al.* 2016) or reduced accessibility of Old soil C pools due to greater C adsorption to minerals with lower water content under lower moisture conditions (Conant *et al.* 2011). It is also possible that the chemical quality of remaining Old soil C differed for warmed and unwarmed treatments,

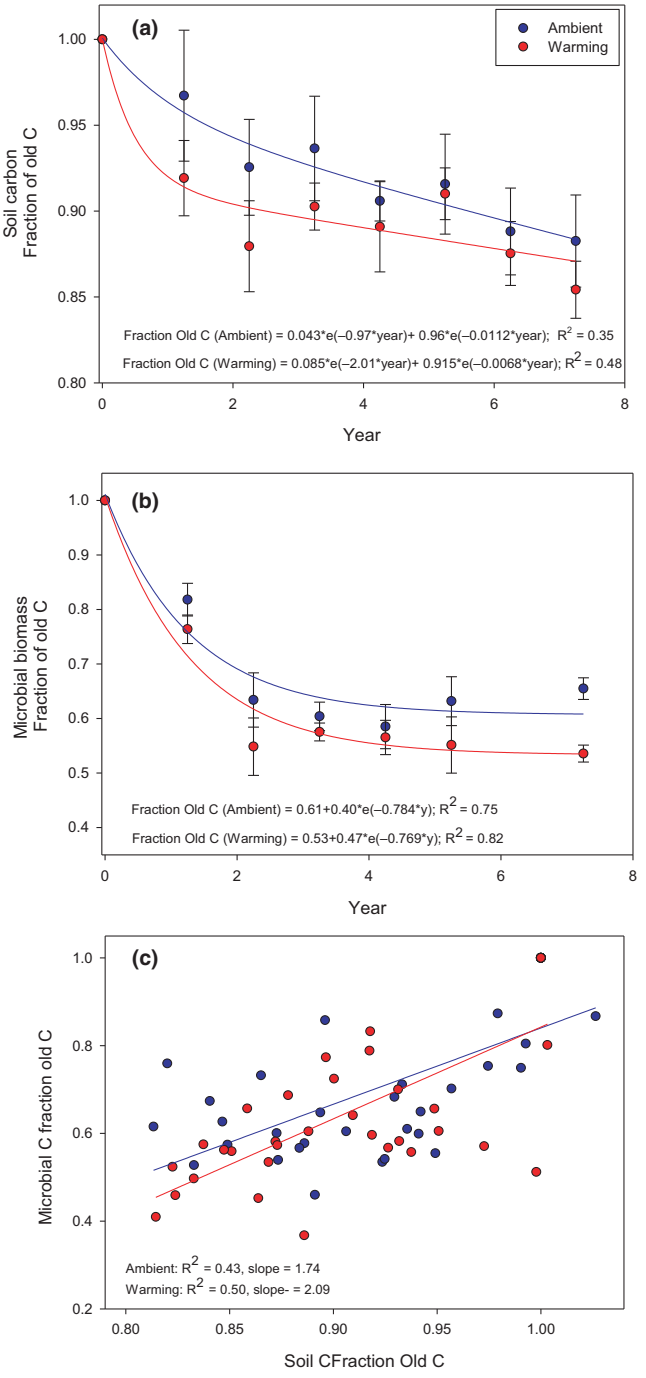

Figure 4 Dynamics of the fraction of Old C under warming and control conditions over time at the PHACE experiment in soil (a) and microbial biomass (b) and the relationship between these two (c). Fractions derived from isotopic partitioning of FACE (Free Air CO_2 Enrichment) C vs. existing C (see Methods). Time zero is the start of the CO_2 treatment in April 2006. Values in a and b are averages of 0–15 soil with standard error (n = 5), values in **c** are individual plots.

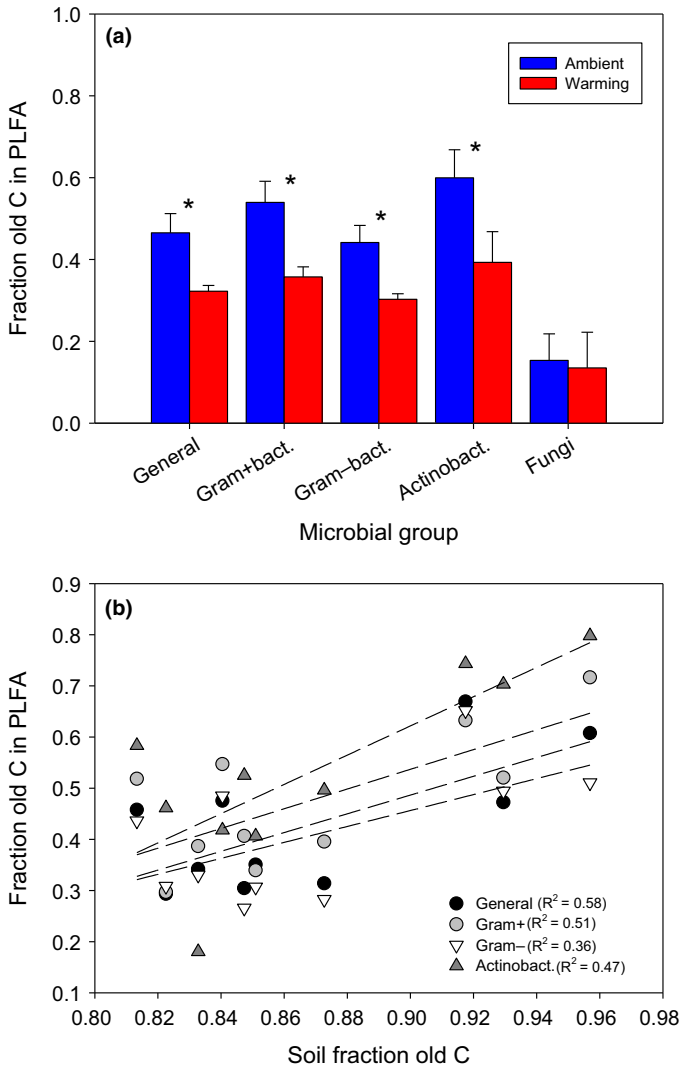

Figure 5 The fraction of Old soil C in PLFA markers of microbial functional groups and general microbial markers under warming and ambient temperature conditions after 7.25 years of warming (a) and their linear relationships with the fraction of Old C in soil (b). Asterisks indicate significant difference (t-test, $P < 0.05$) between warming and Ambient temperature treatments. Values in (a) are means with standard error (n = 5). Points in (b) represent individual experimental plots. Fit lines and R^2 from linear regressions that were significant ($p < 0.06$). Fractions derived from isotopic partitioning of FACE (Free Air CO_2 Enrichment) C vs. existing C (see Methods).

for example, due to labile substrate depletion with warming (Tucker *et al.* 2013; Pold *et al.* 2017).

Sustained isotopic tracking of soil C in field conditions revealed responses that would not have been apparent otherwise. Our empirical findings suggested that even for water-limited systems, soil moisture and its impacts on decomposition and plant inputs alone cannot explain the interactive effects of climate changes on soil C pools and their use by microbes. The dependency of the impacts of warming on CO_2 conditions demonstrates the need for multifactorial studies as well as the importance of including mechanisms that could account for interactive effects of climate factors in future model development. More specifically, our findings indicated the importance of potential CO_2 effects on the susceptibility of soil C to decomposition and differential warming effects on soil C pools. Our observations also support the value of considering the differential responses of distinct microbial groups to warming in relation to soil C pool responses. We

conclusively determined that warming negatively affected the total and Old C pools, and the use of Old C by microbes but only under future high CO_2 conditions. These findings call for caution extending predictions of warming impacts based on observations under contemporary CO_2 conditions. Our observations should be valuable for incorporating microbial function into models, and for realistic C model parameterisation, as the C pools studied are fully measurable (Luo *et al.* 2016).

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AUTHORSHIP

YC, FD, EP, DL and DB conducted the research. YC analysed the data and wrote the manuscript with contributions from other authors.

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