Radiocarbon analysis of soil microbial biomass via direct chloroform extraction

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Abstract

Microbial processing of soil organic matter is a significant driver of C cycling, yet we lack an understanding of what shapes the turnover of this large terrestrial pool. In part, this is due to limited options for accurately identifying the source of C assimilated by microbial communities. Laboratory incubations are the most common method for this; however, they can introduce artifacts due to sample disruption and processing and can take months to produce sufficient CO_2 for analysis. We present a biomass extraction method which allows for the direct ^{14}C analysis of microbial biomolecules and compare the results to laboratory incubations. In the upper 50 cm soil depths, the $\Delta^{14}C$ from incubations was indistinguishable from that of extracted microbial biomass. Below 50 cm, the $\Delta^{14}C$ of the biomass was more depleted than that of the incubations, either due to the stimulation of labile C decomposition in the incubations, or the inclusion of biomolecules from non-living cells in the biomass extractions. Our results suggest that measurement of $\Delta^{14}C$ of microbial biomass extracts can be a useful alternative to soil incubations, possibly avoiding some of the drawbacks associated with laboratory incubations.

Keywords

Soil microbial biomass, Radiocarbon, Soil organic carbon, Soil incubation, Direct chloroform extraction

Introduction

Soils are a significant component of the Earth's carbon (C) cycle (Eswaran et al. 1993, Batjes 1996, Jobbágy and Jackson 2000), yet a mechanistic understanding of what controls the turnover of this large C pool remains elusive. Soil organic C (SOC) stocks are primarily controlled by the balance of plant-derived C inputs and subsequent CO_2 efflux due to microbial decomposition and root respiration (Davidson and Janssens 2006). Microbial respiration of organic C accounts for roughly half of the total CO_2 production from soils (Yan et al. 2018), though this number varies with ecosystem type, temperature, and moisture (Subke et al. 2006). The SOC used by microorganisms therefore has a significant impact on soil C cycling, influencing what SOC is cycled rapidly versus left to persist for centuries to millennia.

Radiocarbon (^{14}C) is the gold standard for determining both the age and turnover rate of soil C, providing an invaluable metric for evaluating long-term C stability. Given the importance of microbial SOC cycling, many studies use laboratory soil incubations to measure the rate of heterotrophic respiration and the $\Delta^{14}C$ of respired CO_2 to assess C turnover utilization by microbes. While incubations provide an integrated assessment of microbial respiration and C turnover, soil sampling and preparation prior to incubation can result in artifacts due to the disruption of soil structure, roots, and microbial communities (Salomé et al. 2010, Herbst et al. 2016, Schädel et al 2020, Patel et al. 2022). Comparisons between field-based and laboratory incubation studies show differences in gas flux rates (Williams et al. 1998, Patel et al. 2022, Risk et al. 2008) and younger respired C in the field (Phillips et al. 2013), suggesting that additional methods to assess microbial processes would be valuable.

To date, very few techniques other than laboratory incubations have been developed to specifically measure the $\Delta^{14}C$ of organic C used by microbial communities. The only existing alternatives have relied on modifying the traditional chloroform fumigation extraction (CFE) approach–conducted by fumigating a soil with chloroform and then extracting the released biomolecules using a salt solution (Vance et al. 1987). With CFE, the quantity of C is compared to a control extraction conducted without chloroform; the difference between the two is a measure of the total microbial biomass. Fearing that chloroform C contamination might render natural abundance ^{14}C analysis impractical, Rumpel et al. 2001 opted to rupture microbial cells using freeze-drying cycles rather than chloroform. However, Garnett et al., 2011 successfully used the traditional CFE protocol and found the chloroform C contamination was manageable, however their method requires a specialized vacuum system.

A more quantitative estimate of the age and turnover time of various soil organic pools is a key prerequisite to more accurate modeling of the stability of SOM under varying edaphic conditions. Here, we report on a new microbial biomass extraction method for ^{14}C analysis, allowing for the empirical measurement of microbially assimilated C. The method is based on direct chloroform extraction which applies chloroform directly to the soil (Gregorich et al 1990, Setia et al. 2012, Slessarev et al. 2020). We compare the results of our ^{14}C biomass extraction method to those of a traditional laboratory incubation from a soil profile to evaluate the utility of the method and future applications. Additionally, we evaluate the ^{14}C blank contribution of our chloroform extraction protocol using a size series of ^{14}C modern and fossil standards.

Methods

Soil sampling, storage, and bulk soil analysis

The soil samples used in this study were collected from the University of California Hopland Research and Extension Center in Hopland, CA in January 2022 (39.001º, -123.069º). The mean annual temperature and precipitation at the site are 15˚C and 940 mm/y, respectively, and the soil is classified as a Typic Haploxeralf with sandstone and shale parent material (Foley et al. 2022, Fossum et al. 2022). Samples were collected from a soil pit face at depth increments of 0 – 10 cm, 10 – 20 cm, 20 – 50 cm, and 50 – 100 cm. One aliquot of each sample was sealed in a bag and left at room temperature until processing for laboratory incubations. A second aliquot of each sample was sealed in a bag and kept at 4 ºC until use in microbial biomass extractions. Upon returning from the field, a subsample of bulk soil from each depth was air dried, sieved to 2 mm, and then ground in a ball mill. Triplicate samples of the ground bulk soil were sealed into quartz tubes for ^{14}C and $\delta^{13}C$ analysis, respectively.

Laboratory soil incubations

For each depth increment, three technical replicates were incubated. Between 90 and 200 g of soil was placed in a 32 oz jar after carefully removing visible roots with tweezers. Soil aggregates were intentionally left intact to minimize disturbance of the soil structure. After a 24 h pre-incubation at room temperature, the jars were flushed with > 4 times the headspace volume with certified CO_2-free air and sealed. Incubations were conducted in triplicate from each depth increment and sampled periodically to determine headspace CO_2 concentration via a LI-830 (LI-COR) infrared gas analyzer. After reaching ~1% CO_2, the headspace was transferred from each jar into a glass flask and immediately purified and graphitized for ^{14}C analysis. The duration of

incubation was dependent on the rate of CO_2 respiration and ranged between 5 days for surface soils to 47 days for the deepest samples.

Microbial biomass extraction and calculations

Microbial soil biomass was extracted and quantified based on a modified direct extraction method from Setia et al. (2012). Two technical replicate extractions were done from each soil depth to test the reproducibility of the method. To minimize C contamination, all glassware was acid washed and baked at 400 ºC prior to use. 25 g of 2 mm sieved, field moist soil was weighed into glass flasks along with 100 mL of Ultrapure water. For each replicate, two soil slurries were prepared. 2.5 mL of ethanol-free chloroform (Alfar Aesar, L14759) was added to one soil slurry, producing one "water" and one "chloroform" extract for each soil sample. The flasks were capped with glass stoppers and shaken in an orbital motion for 1 h at 140 RPM. The samples were vacuum filtered through pre-baked 0.7 μm glass fiber filters, after which the filtrate was bubbled vigorously with ultra-high purity N_2 for 30 m to remove any residual chloroform. N_2 was introduced via pre-baked glass pipettes secured to a nitrogen evaporator.

The extraction process was repeated three times for soil samples collected from depths below 20 cm to recover enough C for ^{14}C analysis. Extracts were finally filtered through a 0.2 μm polycarbonate filter to remove visible soil particles. A split of each sample was reserved for total organic carbon (TOC) analysis and the remainder was concentrated in an evaporative centrifuge. The concentrated biomass extracts were transferred to pre-baked 6 mm quartz tubes using 0.01 M HCl then dried to completion. CuO and Ag powder were added, and the sample tubes were loaded into 9 mm quartz tubes, evacuated, sealed, and combusted at 900 ºC. The quantity of the

125 microbial biomass was calculated by subtracting the total organic C content of the water extract
126 from the chloroform extract, and the $\Delta^{14}C$ of the microbial biomass (MB) extract was calculated
127 using (Garnett et al. 2011):
128 $\Delta^{14}C_{MB} = (\Delta^{14}C_C * C_C - \Delta^{14}C_W * C_W) / (C_C - C_W)$ Eq. 1
129 where $\Delta^{14}C_C$ and $\Delta^{14}C_W$ refer to the measured ^{14}C concentration of the chloroform and water,
130 and C_C and C_W represent the mass of carbon in the chloroform and water extracts, respectively.
131
132 *Blank assessment and $F^{14}C$ data correction*
133 To assess the C contamination (blank) introduced during the microbial biomass exactions, a size
134 series of ^{14}C-modern and -dead material (ANU sucrose and alanine, respectively) were processed
135 in an identical fashion to the soil samples, in the range of 40 to 150 μg C. The size and fraction
136 modern ($F^{14}C$) of the blank were then determined using the methods and published R script from
137 Sun et al. (2020). Briefly, a Bayesian model was used to fit thousands of linear regression lines
138 between the $F^{14}C$ and inverse of the sample size (1/μg C), allowing for the calculation of the
139 $F^{14}C$ and size of the blank, as well as their associated uncertainties. The R script was run in R
140 Studio version 4.1.2 (R Core Team, 2021). The calculated blank was then used to correct the
141 measured $F^{14}C$ of the water and chloroform extracts.
142
143 *Sample graphitization and isotopic analyses*
144 Graphitization and accelerator mass spectrometry (AMS) measurements were conducted at the
145 Center for Accelerator Mass Spectrometry (CAMS) at Lawrence Livermore National
146 Laboratory. Bulk soil samples and microbial biomass extracts were prepared for graphitization
147 through sealed-tube combustion at 900 ºC in an evacuated quartz tube in the presence of CuO

and Ag. The CO_2 produced from sealed-tube combustion, as well as the headspace CO_2 from the incubations, was purified and then reduced to graphite at 570 ºC in the presence of iron powder and H_2 (Vogel et al. 1984). Samples were run on the model FN Van de Graaff AMS system at CAMS. During purification of the CO_2, a split of each of the incubation and microbial biomass samples was taken and subsequently sent to the Stable Isotope Geosciences Facility at Texas A&M University for $\delta^{13}C$ analysis on a Thermo Scientific MAT 253 Dual Inlet Stable Isotope Ratio Mass Spectrometer. Bulk soil samples were measured for % C and $\delta^{13}C$ at the Center for Stable Isotope Biogeochemistry, University of California, Berkeley on a CHNOS Elemental Analyzer interfaced to an IsoPrime100 Isotope Ratio Mass Spectrometer. Measured radiocarbon values were corrected using offline $\delta^{13}C$ values and reported as age-corrected $\Delta^{14}C$ following the conventions of Stuiver and Polach, 1977.

Statistical analysis

Statistical analyses were conducted in R Studio version 4.1.2 (R Core Team, 2021). Analysis of Variance (ANOVA) was used to test for significant differences in $\Delta^{14}C$ value between incubation or biomass extraction at each depth.

Results and discussion

To assess the reliability and variance of the direct chloroform microbial biomass extraction, we compared $\Delta^{14}C$ values of calculated microbial biomass from two replicate extractions to the $\Delta^{14}C$ values of respired CO_2 from three replicate incubations at each depth increment. Regardless of depth increment, the variance of $\Delta^{14}C$ values from technical replicate soil incubations (n=3) was

171 less than that of replicate biomass extractions (n=2), and the variability was larger at depth for
172 both methods (Tables 1-2, Fig. 1). In the upper 50 cm, the average $\Delta^{14}C$ of respired CO_2 was not
173 significantly different than the $\Delta^{14}C$ of the microbial biomass extract ($p > 0.05$) (Fig. 1). Below
174 50 cm, the respired CO_2 was significantly less depleted than the extracted biomass ($p < 0.01$).
175 The average $\Delta^{14}C$ of respired CO_2 from the 0-10, 10-20, 20-50, and 50-100 cm depths was 6 ± 5,
176 17 ± 4, -3 ± 10, and -48 ± 17‰ (± SD, n=3) (Table 1, Fig.1), and the average $\Delta^{14}C$ of extracted
177 microbial biomass was 14 ± 17, 15 ± 10, 21 ± 22, and -220 ± 53‰ (± SD, n=2) (Table 2, Fig. 1).
178
179 We conducted a blank assessment by extracting a series of ^{14}C-modern and -dead materials.
180 From this blank assessment, we estimated that the biomass extraction protocol introduced 2.22 ±
181 0.40 μg C with a $F^{14}C$ value of 0.36 ± 0.08. Measured $F^{14}C$ values and AMS target sizes for the
182 samples used in the blank assessment size series can be found in Supplemental Table 1.
183
184 *Comparison of biomass extraction and laboratory incubation methods*
185 We found that both incubation and chloroform extraction methods of estimating microbial
186 biomass C produced similar $\Delta^{14}C$ results in the upper 50 cm soil increment, indicating that for
187 these surface soils, either method could be used to assess microbially used C. In contrast, the
188 $\Delta^{14}C$ values for soil collected from below 50 cm from the two methods diverge. It is possible that
189 the soil sampling process and sample handling prior to incubation released fresh, labile C that
190 otherwise would not have been accessible for decomposition (Salomé et al. 2010, Herbst et al.
191 2016, Schädel et al 2020, Patel et al. 2022). Alternatively, the ^{14}C depleted biomass values in the
192 deeper soils may reflect non-living cell material that was liberated by the chloroform biomass
193 extraction. This method should release all membrane-contained biomolecules from the soil,

194 including microbial necromass and lipids, which previous reports suggest are the most persistent
195 and ^{14}C depleted compound class in soil (van der Voort et al. 2017, Gies et al. 2020). As
196 microbial community abundance and activity declines with depth, the proportion of these
197 biomolecules associated with inactive or previously lysed cells is likely to become more ^{14}C
198 depleted and comprise a larger proportion of the total biomass extract. A better understanding of
199 what molecules comprise this deep biomass C pool should be explored in future work.
200
201 Due to the natural decrease in microbial activity at depth, it can be difficult to produce enough C
202 for a robust AMS measurement using either incubation or extraction methods. Even with a large
203 mass of soil, soil incubations often need to run for months during which time microbial
204 community diversity may shift, creating artifacts and biasing the results, and lengthy experiments
205 can be problematic for some researchers (Schädel et al. 2020). For the chloroform biomass
206 extraction method, the issue of low C recovery at depth can be circumvented by extracting from
207 a larger soil mass, thereby increasing the amount of extracted biomass. However, scaling up the
208 extraction also increases the amount of active time required to process the sample. We found that
209 simply doubling the amount of soil and water/chloroform in a single extraction significantly
210 reduced the rate of filtration. Instead, we opted to pool extracts from multiple separate
211 extractions, thereby maintaining a standard time and filter volume for each extraction. While we
212 were able to identify and eliminate some sources of ^{14}C contamination, we were unsuccessful in
213 completely eliminating it. We hypothesize that some contribution to the blank may originate
214 from the polycarbonate filter used to remove fine particles (0.2 μm). Binder-free glass fiber
215 filters at this pore size were not available, however it is possible that removal of these fine
216 particles is not worth the added contamination.

Conclusions

Understanding the role of microbial communities in soil C cycling and the persistence of soil organic matter is challenging given the heterogenous and complex nature of soils. While natural abundance ^{14}C laboratory incubations have some drawbacks, they have provided valuable insight into microbial decomposition and assimilation of soil C. However, additional methods are needed to provide a more direct and mechanistic understanding of microbial C assimilation. The ^{14}C chloroform biomass extraction method we present here can be a useful alternative to soil incubations, possibly avoiding some of the artifacts associated with incubations, though additional research will be needed to assess the inclusion of non-living cells during biomass extraction. Additional methods for isolating specific, short-lived biomolecules, such as RNA, may be required to unambiguously determine the $\Delta^{14}C$ of organic molecules being assimilated by active microbial communities.

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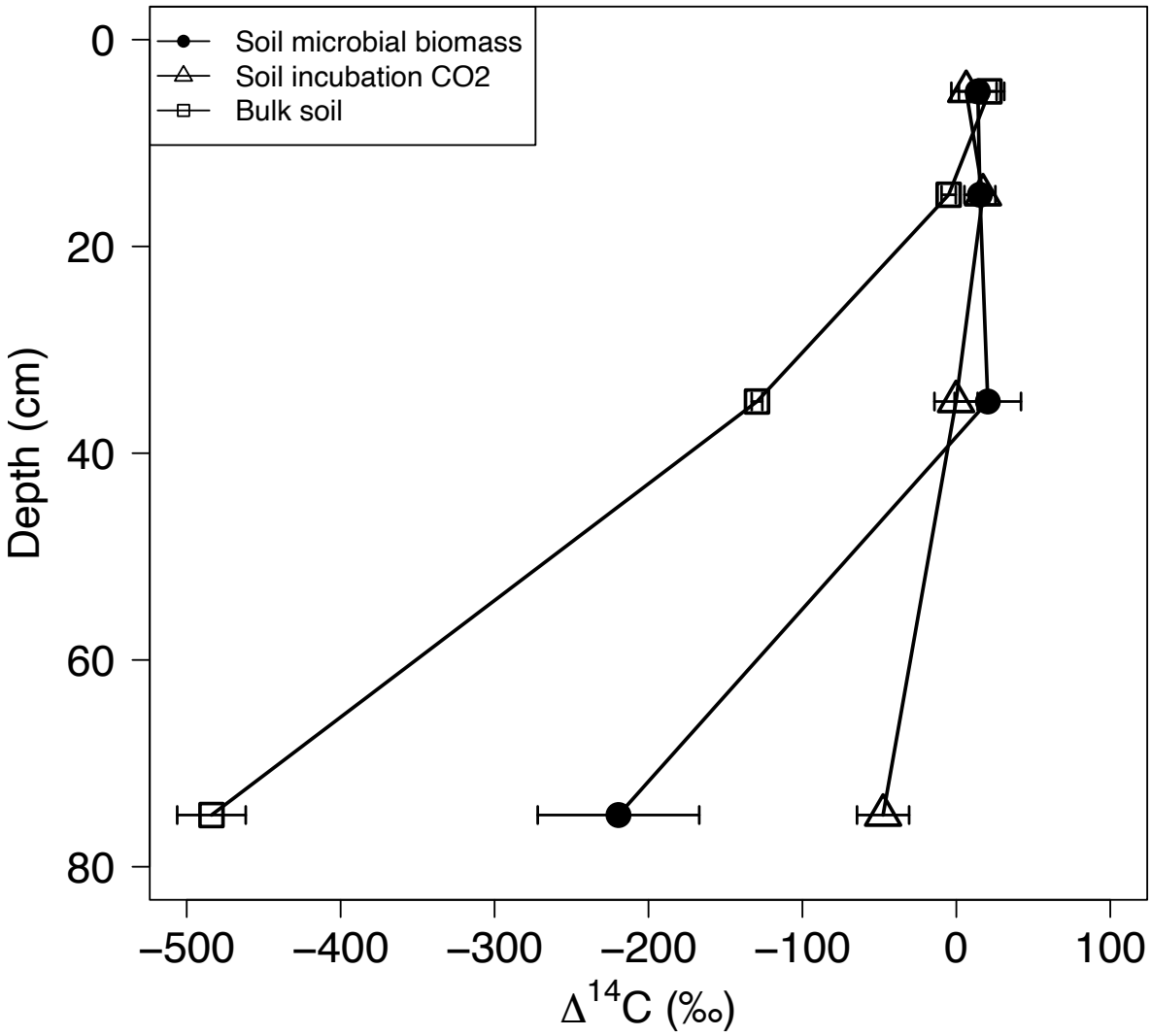
352 **Figures and Tables**



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354 **Figure 1.** Average $\Delta^{14}C$ value of bulk soil (n=3), respired CO_2 from laboratory soil incubations
355 (n=3), and soil microbial biomass from direct chloroform extraction (n=2) from a Hopland, CA
356 annual grassland soil, sampled over four depth increments. Error bars indicate standard deviation
357 of replicates.

CAMS ID	Soil depth (cm)	Technical replicate	$\delta^{13}C$ (‰)	$F^{14}C \pm err$	$\Delta^{14}C \pm err$ (‰)
188227	0-10	A	-30.0	1.0124 ± 0.0032	4 ± 3
188228	0-10	B	-29.9	1.0126 ± 0.0032	4 ± 3
188229	0-10	C	-30.0	1.0207 ± 0.0044	12 ± 4
188230	10-20	A	-29.4	1.0229 ± 0.0032	14 ± 3
188231	10-20	B	-29.4	1.0252 ± 0.0034	16 ± 3
188232	10-20	C	-29.5	1.0305 ± 0.0035	22 ± 4
188233	20-50	A	-28.2	1.0027 ± 0.0033	-6 ± 3
188234	20-50	B	-28.0	0.9983 ± 0.0032	-10 ± 3
188392	20-50	C	-30.0	1.0170 ± 0.0036	8 ± 4
188388	50-100	A	-25.6	0.9418 ± 0.0028	-66 ± 3
188389	50-100	B	-26.6	0.9748 ± 0.0029	-34 ± 3
188390	50-100	C	-26.2	0.9657 ± 0.0029	-43 ± 3

Table 1. Radiocarbon values and measurement error of CO_2 respired from triplicate laboratory incubations of a grassland soil collected over four depth increments from Hopland, CA.

CAMS ID	Extract type	Technical replicate	Soil depth (cm)	$F^{14}C \pm err$	$\Delta^{14}C \pm err$ (‰)
189091	Water	A	0-10	1.0274 ± 0.0040	18 ± 4
189090	Chloroform	A	0-10	1.0133 ± 0.0030	5 ± 3
-	Biomass	A	0-10	-	2
189204	Water	B	0-10	1.0081 ± 0.0099	-1 ± 10
189203	Chloroform	B	0-10	1.0334 ± 0.0036	24 ± 4
-	Biomass	B	0-10	-	26
189093	Water	A	10-20	1.0173 ± 0.0063	8 ± 6
189092	Chloroform	A	10-20	1.0243 ± 0.0036	15 ± 4
-	Biomass	A	10-20	-	22
189206	Water	B	10-20	0.9872 ± 0.0083	21 ± 8
189205	Chloroform	B	10-20	1.0108 ± 0.0037	2 ± 4
-	Biomass	B	10-20	-	8
189099	Water	A	20-50	0.9294 ± 0.0050	-79 ± 5
189098	Chloroform	A	20-50	0.9858 ± 0.0036	-23 ± 4
-	Biomass	A	20-50	-	5
189212	Water	B	20-50	0.9003 ± 0.0056	-108 ± 6
189211	Chloroform	B	20-50	1.0138 ± 0.0031	5 ± 3
-	Biomass	B	20-50	-	36
189101	Water	A	50-100	0.7728 ± 0.0058	-234 ± 6
189100	Chloroform	A	50-100	0.7641 ± 0.0034	-243 ± 3
-	Biomass	A	50-100	-	-257
189214	Water	B	50-100	0.5911 ± 0.0073	-414 ± 7
189213	Chloroform	B	50-100	0.6988 ± 0.0063	-307 ± 6
-	Biomass	B	50-100	-	-182

Table 2. Blank corrected measured radiocarbon values and measurement error of water and chloroform extracts and the calculated $\Delta^{14}C$ of microbial biomass (Eq.1) from a grassland soil collected at four depth increments in Hopland, CA.

CAMS ID	Soil depth (cm)	Technical replicate	$F^{14}C \pm err$	$\Delta^{14}C \pm err$ (‰)	C (%)	$\delta^{13}C$ (‰)
189696	0-10	A	1.0252 ± 0.0031	16 ± 3	2.49	-27.88
189697	0-10	B	1.0317 ± 0.0031	23 ± 3	2.66	-28.13
189698	0-10	C	1.0344 ± 0.0034	25 ± 3	2.33	-27.68
189699	10-20	A	0.9987 ± 0.0030	-10 ± 3	1.68	-26.73
189700	10-20	B	1.0080 ± 0.0030	-1 ± 3	1.58	-26.79
189701	10-20	C	1.0044 ± 0.0030	-4 ± 3	1.4	-26.69
189429	20-50	A	0.8819 ± 0.0039	-126 ± 4	0.44	-25.17
189430	20-50	B	0.8770 ± 0.0041	-130 ± 4	0.55	-25.53
189431	20-50	C	0.8751 ± 0.0040	-133 ± 4	0.47	-25.28
189432	50-100	A	0.5232 ± 0.0027	-481 ± 3	0.25	-23.86
189433	50-100	B	0.5417 ± 0.0027	-463 ± 3	0.24	-23.99
189434	50-100	C	0.4970 ± 0.0026	-507 ± 3	0.24	-24.04

Supplemental table 1. Bulk soil carbon isotopic values of three technical replicates from a grassland soil in Hopland, CA collected at four depth increments.

Type	CAMS ID	Mass (µg C)	Measured $F^{14}C$ ± err
Modern	189970	47	1.4594 ± 0.0060
Modern	189973	65	1.4689 ± 0.0042
Modern	189974	73	1.4703 ± 0.0043
Modern	189975	119	1.4591 ± 0.0042
Modern	189976	125	1.4670 ± 0.0042
Modern	189972	127	1.4729 ± 0.0042
Modern	189971	135	1.4481 ± 0.0046
Modern	189977	223	1.4656 ± 0.0042
Modern	189978	247	1.4779 ± 0.0043
Modern	189980	462	1.4670 ± 0.0039
Modern	189979	473	1.4697 ± 0.0042
Modern	189087	478	1.4816 ± 0.0043
Modern	189086	499	1.4723 ± 0.0043
Modern	189982	504	1.4667 ± 0.0042
Modern	189981	506	1.4716 ± 0.0042
Modern	189208	535	1.4972 ± 0.0043
Modern	189207	574	1.4877 ± 0.0043
Modern	189215	613	1.4902 ± 0.0043
Modern	189216	683	1.4826 ± 0.0045
Modern	189095	823	1.4838 ± 0.0053
Modern	189094	855	1.4784 ± 0.0053
Dead	189985	39	0.0244 ± 0.0006
Dead	189986	39	0.0216 ± 0.0004
Dead	189988	65	0.0251 ± 0.0004
Dead	189984	83	0.0204 ± 0.0004
Dead	189989	83	0.0241 ± 0.0004
Dead	189990	83	0.0175 ± 0.0003
Dead	189983	91	0.0257 ± 0.0006
Dead	189209	408	0.0102 ± 0.0001
Dead	189210	519	0.0068 ± 0.0001
Dead	189089	558	0.0104 ± 0.0002
Dead	189097	709	0.0077 ± 0.0002
Dead	189096	758	0.0095 ± 0.0002
Dead	189088	787	0.0085 ± 0.0002
Dead	189217	894	0.0093 ± 0.0001
Dead	189218	940	0.0079 ± 0.0001

Supplemental Table 2. Measured radiocarbon value and sample mass of ^{14}C-modern and -dead standards used for ^{14}C blank assessment of the direct chloroform extraction procedure.