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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, the inclusion of biomolecules from non-living cells in the biomass extractions, or differences in C used for assimilation versus respiration. Our results suggest that measurement of $\Delta^{14}C$ of microbial biomass extracts can be a useful alternative to soil incubations.

KEYWORDS: direct chloroform extraction, radiocarbon, soil incubation, soil microbial biomass, soil organic carbon.

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 (Bond-Lamberty et al. 2004), 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; Risk et al. 2008; Patel

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et al. 2022) 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 (39.001°, -123.069°). 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 on Cretaceous sandstone and shale (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 for one week until processing for laboratory incubations. A second aliquot of each sample was sealed in a bag and kept at 4°C until use in microbial biomass extractions, approximately three months. 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

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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 400ºC for 5 hr 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 sample, two soil slurries were prepared. 2.5 mL of ethanol-free chloroform (99%+ chloroform with ca 50 ppm amylene, 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 (400ºC for 5 hr), after which the filtrate was bubbled vigorously with Ultra-High Purity N_2 (99.999%) for 30 min to remove any residual chloroform. N_2 was introduced via pre-baked glass pipettes secured to a nitrogen evaporator. Extracts were finally filtered through a 0.2 μm polycarbonate filter to remove visible soil particles. For samples below 20 cm, extracts from three separate 25 g "water" or "chloroform" samples were pooled to recover sufficient C for ^{14}C analysis, totaling 75 g of material.

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 (900ºC for 5 hr) 6 mm quartz tubes using 0.01 M HCl to remove any inorganic carbon, 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 900ºC (Trumbore et al. 2016). The quantity of the microbial biomass was calculated by subtracting the total organic C content of the water extract from the chloroform extract, and the $\Delta^{14}C$ of the microbial biomass (MB) extract was calculated using (Garnett et al. 2011):

$$\Delta^{14}C_{MB} = \left(\Delta^{14}C_C * C_C - \Delta^{14}C_W * C_W\right)/(C_C - C_W) \quad (1)$$

where $\Delta^{14}C_C$ and $\Delta^{14}C_W$ refer to the measured ^{14}C concentration of the chloroform and water, and C_C and C_W represent the mass of carbon in the chloroform and water extracts, respectively.

Blank Assessment and $F^{14}C$ Data Correction

To assess the C contamination (blank) introduced during the microbial biomass exactions, a size series of ^{14}C-modern and -dead material (ANU sucrose and alanine, respectively) were processed in an identical fashion to the soil samples, in the range of 40 to 150 μg C. In total, 21 modern and 15 dead samples were analyzed in the size series. The size and fraction modern ($F^{14}C$) of the blank were then determined using the methods and published R script from Sun et al. (2020). Briefly, a Bayesian model was used to fit thousands of linear regression lines between the $F^{14}C$ and inverse of the sample size (1/μg C), allowing for the calculation of the $F^{14}C$ and size of the blank, as well as their associated uncertainties. The R script was run in R Studio version 4.1.2 (R Core Team 2021). The calculated blank was then used to correct the measured $F^{14}C$ of the water and chloroform extracts.

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Sample Graphitization and Isotopic Analyses

Graphitization and accelerator mass spectrometry (AMS) measurements were conducted at the Center for Accelerator Mass Spectrometry (CAMS) at Lawrence Livermore National Laboratory. Bulk soil samples and microbial biomass extracts were prepared for graphitization through sealed-tube combustion at 900ºC 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 570ºC 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 $\Delta^{14}C$ using the following equation and conventions from Stuiver and Polach, 1977:

$$\Delta^{14}C = \left(\frac{A_{SN}\, e^{\lambda(1950-x)}}{A_{ON}} - 1\right) * 1000 \quad (2)$$

where A_{SN} is the normalized sample specific activity, A_{ON} is the normalized standard specific activity, λ is 1/8267 yr^{-1}, and x is the year of measurement.

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 (Figure 1; Tables 1–2). Regardless of depth increment, the variance of $\Delta^{14}C$ values from technical replicate soil incubations (n=3) was less than that of replicate biomass extractions (n=2), and the variability was larger at depth for both methods (Tables 1–2; Figure 1). In the upper 50 cm, the average $\Delta^{14}C$ of respired CO_2 was not significantly different than the $\Delta^{14}C$ of the microbial biomass extract ($p > 0.05$) (Figure 1). Below 50 cm, the respired CO_2 was significantly less depleted than the extracted biomass ($p < 0.01$). 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, 17 ± 4, –3 ± 10, and –48 ± 17‰ (± SD, n=3) (Table 1; Figure 1), and the average $\Delta^{14}C$ of extracted microbial biomass was 14 ± 17, 15 ± 10, 21 ± 22, and –220 ± 53‰ (± SD, n=2) (Table 2; Figure 1).

We conducted a blank assessment by extracting a series of ^{14}C-modern and -dead materials. From this blank assessment, we estimated that the biomass extraction protocol introduced 2.22 ± 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 samples used in the blank assessment size series can be found in Supplemental Table 1.

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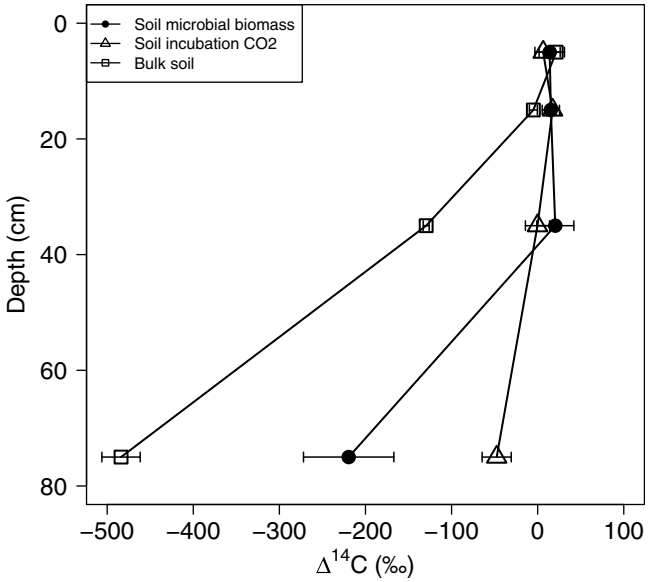

Figure 1 Average $\Delta^{14}C$ value of bulk soil (n=3), respired CO_2 from laboratory soil incubations (n=3), and soil microbial biomass from direct chloroform extraction (n=2) from a Hopland, CA annual grassland soil, sampled over four depth increments. Samples were collected from a soil pit face at depth increments of 0–10 cm, 10–20 cm, 20–50 cm, and 50–100 cm. Error bars indicate standard deviation of replicates.

Comparison of Biomass Extraction and Laboratory Incubation Methods

We found that both incubation and chloroform extraction methods of estimating microbial biomass C produced similar $\Delta^{14}C$ results in the upper 50 cm soil increment (Table 1; Figure 1), indicating that for these surface soils, either method could be used to assess microbially used C. In contrast, the $\Delta^{14}C$ values for soil collected from below 50 cm from the two methods diverge. It is possible that the soil sampling process and sample handling prior to incubation released fresh, labile C that otherwise would not have been accessible for decomposition (Salomé et al. 2010; Herbst et al. 2016; Schädel et al 2020; Patel et al. 2022). Alternatively, the C sources used for respiration and assimilation may differ, which would result in diverging incubation and biomass values. Finally, it is also possible that the ^{14}C depleted biomass values in the deeper soils may reflect non-living cell material that was liberated by the chloroform biomass extraction. This method should release all membrane-contained biomolecules from the soil, including microbial necromass and lipids, which previous reports suggest are the most persistent and ^{14}C depleted compound class in soil (van der Voort et al. 2017; Gies et al. 2021). A better understanding of what molecules comprise this deep biomass C pool should be explored in future work.

Due to the natural decrease in microbial activity at depth, it can be difficult to produce enough C for a robust AMS measurement using either incubation or extraction methods. Even with a large mass of soil, soil incubations often need to run for months during which time microbial community diversity may shift, creating artifacts and biasing the results, and lengthy experiments can be problematic for some researchers (Schädel et al. 2020). For the chloroform

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Table 1 Incubation parameters and isotopic values of heterotrophically respired CO_2 from triplicate laboratory incubations of soil collected at four depths from a grassland in Hopland, CA. Duration of the incubation was driven by respiration rate of individual samples. Incubations ran until sufficient CO_2 had accumulated for radiocarbon analysis.

CAMS ID	Soil depth (cm)	Wet soil mass (mg)	$\delta^{13}C$ (‰)	$F^{14}C \pm$ err	$\Delta^{14}C \pm$ err (‰)	Duration (days)	Day 1 respiration rate (ug C/g soil C/hr)	Day 3 respiration rate (ug C/g soil C/hr)	Day 5 respiration rate (ug C/g soil C/hr)	Total CO_2 collected (mmol)
188227	0–10	99.78	–30.0	1.0124 ± 0.0032	3.6 ± 3.2	5	29.46	29.96	28.90	0.556
188228	0–10	92.03	–29.9	1.0126 ± 0.0032	3.8 ± 3.2	5	26.29	26.30	26.32	0.491
188229	0–10	92.19	–30.0	1.0207 ± 0.0044	11.9 ± 4.4	5	33.29	29.08	31.63	0.529
188230	10–20	110.93	–29.4	1.0229 ± 0.0032	14.0 ± 3.2	7	11.50	11.14	10.97	0.227
188231	10–20	116.11	–29.4	1.0252 ± 0.0034	16.3 ± 3.4	7	12.14	11.66	11.49	0.235
188232	10–20	119.39	–29.5	1.0305 ± 0.0035	21.5 ± 3.5	7	12.61	11.90	11.45	0.211
188233	20–50	169.6	–28.2	1.0027 ± 0.0033	–6.0 ± 3.3	19	11.53	9.71	9.08	0.178
188234	20–50	174.15	–28.0	0.9983 ± 0.0032	–10.3 ± 3.2	19	9.57	7.88	7.33	0.174
188392	20–50	186.96	–30.0	1.0170 ± 0.0036	8.2 ± 3.6	19	11.12	9.60	9.17	0.212
188388	50–100	203.08	–25.6	0.9418 ± 0.0028	–66.4 ± 2.8	34	13.02	8.30	7.14	0.155
188389	50–100	207.45	–26.6	0.9748 ± 0.0029	–33.6 ± 2.9	34	13.20	8.53	7.66	0.158
188390	50–100	214.98	–26.2	0.9657 ± 0.0029	–42.9 ± 2.9	34	11.22	7.36	6.58	0.132

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	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

biomass extraction method, the issue of low C recovery at depth can be circumvented by extracting from a larger soil mass, thereby increasing the amount of extracted biomass. However, scaling up the extraction also increases the amount of active time required to process the sample. We found that simply doubling the amount of soil and water/chloroform in a single extraction significantly reduced the rate of filtration. Instead, we opted to pool extracts from multiple separate extractions, thereby maintaining a standard time and filter volume for each extraction. While we were able to identify and eliminate some sources of ^{14}C contamination, we were unsuccessful in completely eliminating it. We hypothesize that some contribution to the blank may originate from the polycarbonate filter used to remove fine particles (0.2 μm). Binder-free glass fiber filters at this pore size were not available, however testing without these filters resulted in large amounts of colloidal material passing into the filtrate and skewing the $\Delta^{14}C$ values.

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

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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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SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit https://doi.org/10.1017/RDC.2023.80

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