Large impacts of small methane fluxes on carbon isotope values of soil respiration

Wenjuan Huang  
*Iowa State University*, wjhuang@iastate.edu

Steven J. Hall  
*Iowa State University*, stevenjh@iastate.edu

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Abstract
Carbon dioxide isotope (δ13C of CO2) analysis is increasingly used to address a broad range of questions involving soil C dynamics and respiration sources. However, attaining δ13C mass balance is critical for robust interpretation. Many ecosystems exhibit methane (CH4) fluxes that are small in the context of total C budgets, yet may significantly impact δ13C values of CO2 due to large kinetic fractionations during CH4 production. Thus, the δ13C values of CO2 do not directly reflect respiration C sources when co-occurring with CH4, but few studies of terrestrial soils have considered this phenomenon. To assess how CH4 altered the interpretation of δ13C values of CO2, we incubated a Mollisol and Oxisol amended with C4-derived plant litter for 90 days under two headspace treatments: a fluctuating anaerobic/aerobic treatment (four days of anaerobic conditions alternating with four days of aerobic conditions), and a static aerobic treatment (control). We measured δ13C values of CO2 and CH4 with a tunable diode laser absorption spectrometer, using a novel in-line combustion method for CH4. Cumulative δ13C of CO2 differed significantly between treatments in both soils. The δ13C values of CO2 were affected by relatively small CH4 fluxes in the fluctuating anaerobic/aerobic treatment. Effects of CH4 on δ13C values of CO2 were greater in the Oxisol due to its higher percent contribution of CH4 to total C mineralization (18%) than in the Mollisol (3%) during periods of elevated CH4 production. When CH4 accounted for just 2% of total C mineralization, the δ13C values of CO2 differed from total C mineralization by 0.3–1‰, and by 1.4–4.8‰ when CH4 was 10% of C mineralization. These differences are highly significant when interpreting natural abundance δ13C data. Small CH4 fluxes may strongly alter the δ13C values of CO2 relative to total mineralized C. A broad range of mineral and peatland soils can experience temporary oxygen deficits. In these dynamic redox environments, the δ13C values of CO2 should be interpreted with caution and ideally combined with δ13C of CH4 when partitioning sources and mechanisms of soil respiration.

Keywords
Carbon dioxide stable isotopes, Isotope mass balance, Methane isotopic fractionation, Redox fluctuation, Aerobic/anaerobic processes, Wetland

Disciplines
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Large impacts of small methane fluxes
on carbon isotope values of soil respiration

Wenjuan Huang, Steven J. Hall*

Department of Ecology, Evolution, and Organismal Biology, Iowa State University,
251 Bessey Hall, Ames, IA 50011, USA

*Corresponding author.
E-mail address: stevenjh@iastate.edu
ABSTRACT

Carbon dioxide isotope (δ¹³C of CO₂) analysis is increasingly used to address a broad range of questions involving soil C dynamics and respiration sources. However, attaining δ¹³C mass balance is critical for robust interpretation. Many ecosystems exhibit methane (CH₄) fluxes that are small in the context of total C budgets, yet may significantly impact δ¹³C values of CO₂ due to large kinetic fractionations during CH₄ production. Thus, the δ¹³C values of CO₂ do not directly reflect respiration C sources when co-occurring with CH₄, but few studies of terrestrial soils have considered this phenomenon. To assess how CH₄ altered the interpretation of δ¹³C values of CO₂, we incubated a Mollisol and Oxisol amended with C₄-derived plant litter for 90 days under two headspace treatments: a fluctuating anaerobic/aerobic treatment (four days of anaerobic conditions alternating with four days of aerobic conditions), and a static aerobic treatment (control). We measured δ¹³C values of CO₂ and CH₄ with a tunable diode laser absorption spectrometer, using a novel in-line combustion method for CH₄. Cumulative δ¹³C of CO₂ differed significantly between treatments in both soils. The δ¹³C values of CO₂ were affected by relatively small CH₄ fluxes in the fluctuating anaerobic/aerobic treatment. Effects of CH₄ on δ¹³C values of CO₂ were greater in the Oxisol due to its higher percent contribution of CH₄ to total C mineralization (18.22%) than in the Mollisol (3.25%) during periods of elevated CH₄ production. When CH₄ accounted for just 2% of total C mineralization, the δ¹³C values of CO₂ differed from total C mineralization by 0.3 – 1‰, and by 1.4 – 4.8‰ when CH₄ was 10% of C mineralization. These differences are highly significant when interpreting
natural abundance $\delta^{13}C$ data. Small CH$_4$ fluxes may strongly alter the $\delta^{13}C$ values of CO$_2$ relative to total mineralized C. A broad range of mineral and peatland soils can experience temporary oxygen deficits. In these dynamic redox environments, the $\delta^{13}C$ values of CO$_2$ should be interpreted with caution and ideally combined with $\delta^{13}C$ of CH$_4$ when partitioning sources and mechanisms of soil respiration.

**Keywords:** Carbon dioxide stable isotopes; Isotope mass balance; Methane isotopic fractionation; Redox fluctuation; Aerobic/anaerobic processes; Wetland
1. Introduction

Over the recent decades, stable carbon isotope ($\delta^{13}$C) analyses have been extensively used to understand belowground C processes, especially to quantify the sources and dynamics of soil carbon dioxide (CO$_2$) emissions (Amundson et al., 1988; Ehleringer et al., 2000). For instance, measurements of $\delta^{13}$C of CO$_2$ at natural abundance and in $^{13}$C labeling experiments can enable partitioning of heterotrophic and autotrophic respiration (Hanson et al., 2000; Tu and Dawson, 2005), quantification of turnover rates for different soil organic C pools (Collins et al., 2000; Vestergård et al., 2016), and identification of biogeoophysical processes influencing gas dynamics in the soil system (Moyes et al., 2010; Bowling et al., 2015). Robust interpretation of $\delta^{13}$C values of soil respiration is thus important for our understanding of soil and ecosystem C dynamics.

The $\delta^{13}$C values of soil respiration are often thought to reflect $\delta^{13}$C of the substrate from which the CO$_2$ was derived (Ehleringer et al., 2000; Breecker et al., 2015; Hall et al., 2017). However, production of methane (CH$_4$) impacts the interpretation of $\delta^{13}$C values of CO$_2$. When CO$_2$ co-occurs with methane (CH$_4$), the $\delta^{13}$C values of the net CO$_2$ flux may be affected by C isotope fractionation during both methanogenesis and CH$_4$ oxidation (Fig. 1). The fractionation factor ($\varepsilon$) for CH$_4$ production is defined here as: $\varepsilon = ((1000 + \delta^{13}C_C)/(1000 + \delta^{13}C_{CH_4}) - 1) \times 1000 \approx \delta^{13}C_C - \delta^{13}C_{CH_4}$ (Hayes, 1993), where $\delta^{13}C_C$ and $\delta^{13}C_{CH_4}$ are $\delta^{13}$C values of the C source (either CO$_2$ or acetate) and CH$_4$, respectively. During methanogenesis, both the hydrogenotrophic (CO$_2$ reduction; CO$_2$ + 4H$_2$ $\rightarrow$ CH$_4$ + 2H$_2$O) and acetoclastic
(acetate fermentation; \( \text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2 \)) pathways impose large fractionations, with \( \varepsilon \) values of 30 – 90‰ and 7 – 35‰, respectively (Penning et al., 2005; Conrad and Claus, 2009; Blaser and Conrad, 2016). These result in much lower \( \delta^{13} \text{C} \) values in \( \text{CH}_4 \) relative to the C substrate (either \( \text{CO}_2 \) or acetate). By mass balance, residual \( \text{CO}_2 \) from the hydrogenotrophic pathway and \( \text{CO}_2 \) produced by acetate fermentation must be enriched in \( ^{13} \text{C} \) to balance the more depleted \( ^{13} \text{C} \) of \( \text{CH}_4 \) (Whiticar, 1999; Hornibrook et al., 2000). Conversely, \( \text{CH}_4 \) consumption by aerobic (and potentially anaerobic) oxidation preferentially removes isotopically lighter C (\( \varepsilon = 3 – 30\% \); Happell et al., 1994), resulting in higher \( \delta^{13} \text{C} \) values of \( \text{CH}_4 \) and lower \( \delta^{13} \text{C} \) values of \( \text{CO}_2 \) (Fig. 1). Hence, it is clearly important to consider \( \text{CH}_4 \) fractionation effects on \( \delta^{13} \text{C} \) values of \( \text{CO}_2 \). These processes have been reasonably well documented in studies of traditional wetland ecosystems (i.e., consistently saturated soils). For example, previous studies have observed more positive \( \delta^{13} \text{C} \) values of \( \text{CO}_2 \) than bulk soil \( \delta^{13} \text{C} \) in peatlands as a consequence of \( \text{CH}_4 \) production (Corbett et al., 2013; Holmes et al., 2015).

It remains uncertain, however, whether the influence of \( \text{CH}_4 \) on \( \delta^{13} \text{C} \) values of \( \text{CO}_2 \) is also important in terrestrial soils that experience only sporadic or spatially limited \( \text{O}_2 \) deprivation, and correspondingly small net \( \text{CH}_4 \) emissions. According to isotope mass balance, the \( \delta^{13} \text{C} \) value of total mineralized C (\( \text{CO}_2 + \text{CH}_4 \)) can be calculated as:

\[
\delta^{13} \text{C}_{\text{TC}} = \frac{P_{\text{CH}_4}}{100} \times \delta^{13} \text{C}_{\text{CH}_4} + (1 - \frac{P_{\text{CH}_4}}{100}) \times \delta^{13} \text{C}_{\text{CO}_2},
\]

where \( P_{\text{CH}_4} \) is the percentage of \( \text{CH}_4 \) to total mineralized C (hereafter denoted “\( \text{CH}_4 \) percentage”). This equation can be expressed as:

\[
\delta^{13} \text{C}_{\text{TC}} - \delta^{13} \text{C}_{\text{CO}_2} =
\]
\(-P_{\text{CH}_4}/100 \times (\delta^{13}\text{C}_{\text{CO}_2} - \delta^{13}\text{C}_{\text{CH}_4})\) to reflect the impact of \(\text{CH}_4\) on the \(\delta^{13}\text{C}\) value of \(\text{CO}_2\) \((\epsilon_{\text{TC-CO}_2})\). According to the above fractionation factors, the difference between \(\delta^{13}\text{C}\) values of \(\text{CO}_2\) and \(\text{CH}_4\) is expected to vary from 7 to 90‰ in soils under anaerobic conditions, in which hydrogenotrophic and/or acetoclastic methanogenesis occur without any \(\text{CH}_4\) oxidation. However, few studies explored C isotope separation between \(\text{CO}_2\) and \(\text{CH}_4\) \((\delta^{13}\text{C}_{\text{CO}_2} - \delta^{13}\text{C}_{\text{CH}_4})\) in soils with sporadic temporal or spatial \(\text{O}_2\) limitation. Preliminary calculations suggest that small \(\text{CH}_4\) fluxes could significantly impact the interpretation of \(\delta^{13}\text{C}\) values of \(\text{CO}_2\). If we assume a difference in \(\delta^{13}\text{C}\) values of \(\text{CH}_4\) and \(\text{CO}_2\) of 30‰, a 5% contribution of \(\text{CH}_4\) to total mineralized \(\text{C}\) would result in \(\delta^{13}\text{C}\) values of \(\text{CO}_2\) that are 1.5‰ greater than total mineralized \(\text{C}\). This difference would often be highly significant in the context of ecosystem \(\delta^{13}\text{C}\) budgets, where differences < 1‰ can provide insights about local and global \(\text{C}\) cycle processes (Bowling et al., 2014).

Methane production is typically thought to occur under highly reducing conditions that are most prevalent in wetlands or aquatic sediments (Conrad, 1996). However, terrestrial soils can also have low \(\text{O}_2\) concentrations in microsites resulting from imbalances in biological \(\text{O}_2\) consumption relative to diffusive re-supply (Sexstone et al., 1985). Fluctuations in soil \(\text{O}_2\) availability following rain, irrigation, snowmelt, and/or soil frost occur across a broad range of ecosystems including humid forests, grasslands, urban lawns, and croplands (Liptzin et al., 2011; Hall et al., 2013, 2016; Moyes and Bowling, 2013; Jarecke et al., 2016; O’Connell et al., 2018). Temporary depletion of \(\text{O}_2\) and other terminal electron acceptors can provide
favorable conditions for methanogenesis, and both gross and net CH4 production have been shown to occur even in bulk aerobic soils (von Fischer and Hedin, 2007; Liptzin et al., 2011; Yang and Silver, 2016). This implies that it may frequently be necessary to account for trace CH4 production and its δ13C values when using δ13C of CO2 to understand C cycling processes. However, co-occurring measurements of δ13C of CO2 and CH4 from uplands (and even some wetland ecosystems, such as arctic peatlands) remain relatively uncommon.

Here, we incubated a temperate Mollisol and a tropical Oxisol under a fluctuating anaerobic/aerobic condition over 90 days to simulate redox fluctuations driven by variations in moisture and C supply that occur in their natural ecosystem contexts, along with a static aerobic condition (control). We assessed the effects of the fluctuating anaerobic/aerobic treatment on δ13C values of CO2, CH4 and total mineralized C (CO2 + CH4). We hypothesized that the fluctuating anaerobic/aerobic treatment would alter δ13C values of CO2 relative to δ13C of soil mineralized C to a significant extent for ecological interpretation (i.e., one – several ‰) when relatively small CH4 fluxes (PCH4 ~5%) occurred.

2. Materials and methods

2.1. Soil sampling

We sampled a Mollisol and Oxisol characterized by redox fluctuations in March 2017. The Mollisol was from a topographic depression in a field under corn-soybean cultivation in north-central Iowa (41°75′N, 93°41′W), USA, and the Oxisol was from
an upland valley in a perhumid tropical forest near the El Verde field station of the Luquillo Experimental Forest (18°17′N, 65°47′W), Puerto Rico. The Mollisol was formed from till following the Wisconsin glaciation and developed under tallgrass prairie and wetland vegetation. The depression has very poorly drained soils described as mucky silt loam (fine, montmorillonitic, mesic Cumulic Haplaquoll) that experience periodic flooding (Logsdon, 2015). This site was cultivated with corn (Zea mays) and soybean (Glycine max) rotated on an annual basis. The Mollisol was sampled following a corn cultivation phase. We collected soils from the plow layer A horizon (0 – 20 cm), which is mixed via tillage or cultivation every year. Six soil cores (10.2 cm diameter) were sampled in a 50 × 50-m region, and then composited to generate spatially representative samples. The Oxisol was formed from volcaniclastic sediments (Buss et al., 2017). This soil experiences temporal shifts in bulk O2 concentrations, varying from 0% to 21% O2 over scales of hours to weeks (Liptzin et al., 2011). Six replicate soil cores were sampled from the A horizon (0 – 10 cm) of the valley site, composited, and shipped overnight to Iowa State University. We chose to assay the surface A horizons from both soils, given that their rates of anaerobic biogeochemical activity at the surface were higher than in deeper horizons due to greater C availability (Hall et al., 2014; Huang and Hall, 2017).

2.2. Optical $\delta^{13}C$ analysis method

The $\delta^{13}C$ values of CO2 and CH4 are traditionally measured by continuous flow-isotope ratio mass spectrometry. However, relatively low sample throughput and high
costs potentially limit measurement frequency and the capacity to capture temporal variation at short time scales (e.g., hourly – daily) relevant to δ13C dynamics over prolonged experiments (Krüger et al., 2002; Zhang et al., 2012). Alternatively, tunable diode laser absorption spectrometry (TDLAS) has been increasingly used for measuring δ13C values of soil respiration (Marron et al., 2009; Bowling et al., 2015) due to its rapid measurement, low cost and relative analytical simplicity. This method has also been applied to direct measurements of δ13C values of CH4 (Bergamaschi et al., 1994). We recently developed a high-throughput method for measuring δ13C values of CO2 in small gas samples (Hall et al., 2017). Here, we applied a variation of this method to analyze δ13C values of CH4 by adding an in-line CO2 trap and furnace to combust CH4 to CO2, which enabled δ13C measurements of both gases on the same TDLAS instrument using separate replicate samples. This enabled relatively high measurement intensity (total n = 540) compared to previous incubation studies.

2.3. Initial soil chemical analysis

Soil pH (1:2.5 ratio of soil:deionized water) was 8.27 for the Mollisol and 5.03 for the Oxisol. The elevated pH value of the Mollisol indicated the presence of carbonate, which could potentially influence the δ13C values of CO2 following dissolution. We measured carbonate mass and its δ13C values before incubation using a method modified from Amundson et al. (1988). In brief, air-dried and ground subsamples (~0.05 g) of the Mollisol were added to 100-ml bottles capped with Teflon septa sealed with aluminum crimps, and then flushed with ultra-zero (CO2-
free) air for 15 min at 500 mL min⁻¹. Two mL of 3 M HCl was injected to each capped bottle with a gas-tight syringe. The bottles were shaken for 30 min. Five mL of gas collected from the headspace of each bottle was injected to a tunable diode laser absorption spectrometer (TDLAS; TGA200A; Campbell Scientific, Logan, UT, USA) to measure CO₂ concentration and its δ¹³C value (Hall et al., 2017). The carbonate concentration in the Mollisol was 4.75 mg g⁻¹, and its δ¹³C value was -2.08‰.

Bulk soil C and its δ¹³C value were determined to be 38.9 mg g⁻¹ and -19.6‰ in the Mollisol and 44.8 mg g⁻¹ and -28.4‰ in the Oxisol, respectively, measured by an elemental analyzer interfaced with an isotope ratio mass spectrometer (ThermoFinnigan Delta Plus XL, Waltham, MA) at Iowa State University. For the Mollisol, soil organic C was 34.2 mg g⁻¹ after accounting for the carbonate contribution to bulk soil C. A two-source mixing model was used to calculate δ¹³C value of soil organic C (δ¹³C_SOC):

\[ δ¹³C_{SOC} = (C_{bulk} × δ¹³C_{bulk} - C_{carb} × δ¹³C_{carb}) / (C_{bulk} - C_{carb}), \]

where \( C_{bulk} \) and \( C_{carb} \) are the concentrations of bulk C and carbonate, respectively. Thus, the δ¹³C value of SOC in the Mollisol was -22.0‰.

2.4. Laboratory incubation

Soils were gently homogenized after coarse roots and macrofauna were removed. Subsamples of fresh soils (5 g dry mass equivalent) were amended with 0.5 g finely ground leaf tissue of *Andropogon gerardii* (big bluestem, a C₄ grass with a δ¹³C value of -13.3‰) that was harvested shortly after senescence. Litter was added to ameliorate short-term C limitation of microbial metabolism (Chacon et al., 2006). Soil samples
were mixed with litter and deionized water at field moisture capacity (0.46 g H₂O g⁻¹ soil for the Mollisol and 1.01 g H₂O g⁻¹ soil for the Oxisol), which was experimentally determined by saturating soils in the lab and measuring gravimetric water content following 48 hours of drainage. Each replicate soil sample was placed in an open 50 ml centrifuge tube and incubated in a glass jar (946 mL) sealed with a gas-tight aluminum lid equipped with butyl septa for headspace gas purging and sampling.

The soil samples received two headspace treatments: a fluctuating anaerobic/aerobic treatment with four days of N₂ alternating with four days of CO₂-free air, and a static aerobic treatment with CO₂-free air (control). Each treatment had three replicates for both the Mollisol and Oxisol. According to the above treatments, each jar was flushed with the appropriate gas for 15 min at 500 mL min⁻¹ every two days immediately following each headspace gas measurement (described below). Purge gases (CO₂-free air or N₂) were humidified to minimize moisture lost during headspace flushing. Additional water was added as necessary by recording the mass of each sample during the incubation at eight-day intervals. The samples were incubated in the dark at 23 °C for 90 days.

2.5. Gas sample analysis

We collected gas samples for measurements of CO₂ and CH₄ concentrations and their δ¹³C values immediately prior to headspace flushing, enabling us to quantify cumulative gaseous C losses and their δ¹³C values over the entire 90-day experiment. A 5-mL sample was collected via a gas-tight syringe at two-day intervals and directly
injected into the TDLAS via an ultra-zero grade CO$_2$-free air carrier gas to measure the CO$_2$ concentration and its $\delta^{13}$C value (Hall et al., 2017). Gas samples were collected from the fluctuating anaerobic/aerobic treatment at two-day intervals to measure CH$_4$ concentrations by gas chromatography (GC) with a flame ionization detector (GC-2014, Shimadzu, Columbia, MD) and the $\delta^{13}$C of CH$_4$ by TDLAS, as described below. For the control, as the percent contribution of CH$_4$ to C mineralization measured in the static aerobic control averaged only 1.1%, gas samples were collected at four-day intervals to measure CH$_4$ concentrations by GC, and CH$_4$ production over two-day intervals was estimated as the average between consecutive four-day measurements. Here, we did not measure $\delta^{13}$C values of CH$_4$ in the control due to its low contribution of CH$_4$ to C mineralization. Thus, we estimated $\delta^{13}$C values of CH$_4$ in the control using the values measured during the aerobic phase of the fluctuating anaerobic/aerobic treatment when CH$_4$ percentage was equal to or lower than the maximum of the CH$_4$ percentage in the control.

2.6. $\delta^{13}$C values of CH$_4$

We analyzed the $\delta^{13}$C values of CH$_4$ by TDLAS following its oxidation to CO$_2$. The TDLAS sample inlet was connected to ultra-zero grade CO$_2$-free air used as a carrier gas. The gas sample was transported by the carrier gas through soda lime and magnesium perchlorate to remove CO$_2$ and water vapor. The sample then flowed into a ceramic tube in a combustion furnace. The ceramic tube was filled with oxidation catalyst (palladium powder and quartz wool) which quantitatively oxidized CH$_4$ to
CO₂ at ~800 °C (Fisher et al., 2005). The CO₂ was then introduced into the TDLAS to independently measure [¹²C] and [¹³C] mole fractions of CO₂ (Hall et al., 2017). The sample flow rate was 50 mL min⁻¹. Individual gas samples (5 mL) were injected by a polypropylene syringe with a Luer stopcock and a 25-gauge needle.

Three δ¹³C standards for CH₄ (T-iso2, T-iso3 and L-iso1) purchased from Isometric Instruments (Victoria, British Columbia, Canada) were used for calibration. Two of the standards ([CH₄] = 250 μmol mol⁻¹ and δ¹³C value of CH₄ = -38.3 ± 0.2‰ for T-iso3; [CH₄] = 2500 μmol mol⁻¹ and δ¹³C value of CH₄ = -66.5 ± 0.2‰ for L-iso1) and ultra-zero air were used to produce calibration curves for [¹²CO₂] and [¹³CO₂] for calculation of δ¹³C values during post-processing. Multiple samples diluted from the T-iso2 standard ([CH₄] = 2.5% and δ¹³C value of CH₄ = -38.3 ± 0.2‰) by ultra-zero air were analyzed to independently characterize the accuracy and precision of our method. Samples from an additional NIST-traceable standard with [CH₄] = 100.9 μmol mol⁻¹ were combusted over a temperature range of 765 – 840 °C to test the impact of the furnace temperature on measured δ¹³C values of CH₄. To further assess method precision and accuracy, replicates of 32 gas samples were also analyzed for δ¹³C values of CH₄ at the UC Davis Stable Isotope Facility using a ThermoScientific Precon concentration unit interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (ThermoScientific, Bremen, Germany).

2.7. Data processing

The peak heights measured after sample injection on the TDLAS were used for
subsequent calibration and calculation of $\delta^{13}$C values as described in detail in Hall et al. (2017), as calculations based on peak areas yielded lower precision. Peak heights were calculated in reference to the baseline prior to each individual sample.

Relationships between peak heights and known CH$_4$ mole fractions were linear across the range of standards used here (0 – 2500 μmol mol$^{-1}$). Separate linear regressions were used for [$^{12}$CO$_2$] and [$^{13}$CO$_2$] calibration. The $\delta^{13}$C values of CO$_2$ and CH$_4$ were reported in ‰ following the convention: $\delta^{13}C = 1000 \times (R_{\text{Sample}}/RPDB - 1)$, where R is the molar ratio of $^{13}$C/$^{12}$C and RPDB is $^{13}$C/$^{12}$C of the Vienna Pee Dee Belemnite standard.

We calculated the stable C isotope separation between CO$_2$ and CH$_4$ for a given gas sample as follows: $\varepsilon_{\text{CO}_2-\text{CH}_4} = \delta^{13}C_{\text{CO}_2} - \delta^{13}C_{\text{CH}_4}$. The difference between the $\delta^{13}$C values of total mineralized C and CO$_2$ was defined here as $\varepsilon_{\text{TC}-\text{CO}_2} = \delta^{13}C_{\text{TC}} - \delta^{13}C_{\text{CO}_2}$. Thus, $\varepsilon_{\text{TC}-\text{CO}_2}$ can be interpreted as the impact of CH$_4$ on the $\delta^{13}$C value of CO$_2$, which is simplified to $\varepsilon_{\text{TC}-\text{CO}_2} = -P_{\text{CH}_4}/100 \times \varepsilon_{\text{CO}_2-\text{CH}_4}$. The cumulative $\delta^{13}$C values of CO$_2$, weighted by their relative mass fluxes, were calculated as: $\delta^{13}C_{\text{cum}\text{CO}_2} = \sum_{i=0}^{90}(\delta^{13}C_{\text{CO}_2} \times F_{\text{CO}_2})/\sum_{i=0}^{90}F_{\text{CO}_2}$, where $F_{\text{CO}_2}$ is the flux of CO$_2$ over each sampling interval $i$. The cumulative $\delta^{13}$C value of total mineralized C was calculated as: $\delta^{13}C_{\text{cumTC}} = \sum_{i=0}^{90}(\delta^{13}C_{\text{CO}_2} \times F_{\text{CO}_2} + \delta^{13}C_{\text{CH}_4} \times F_{\text{CH}_4})/\sum_{i=0}^{90}(F_{\text{CO}_2} + F_{\text{CH}_4})$, where $F_{\text{CH}_4}$ is the flux of CH$_4$.

2.8. Statistical analysis

We tested the effects of the treatments and different phases of the fluctuating
anaerobic/aerobic treatment (anaerobic versus aerobic) on the $\delta^{13}$C values of CH$_4$, CO$_2$ and total mineralized C, $\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $\varepsilon_{\text{TC-\text{CO}_2}}$ for each soil using a mixed-effects model, in which treatments/phases were treated as a fixed effect and samples as a random effect to account for repeated measurements. Statistically significant differences were accepted at $P < 0.05$. Mean values ± standard deviations were reported throughout the text. All statistical analyses were conducted with the R statistical package (R Core Team, 2018).

3. Results

3.1 Method performance for $\delta^{13}$C value of CH$_4$ measurements by TDLAS

We first verified the accuracy and precision of the $\delta^{13}$C value of CH$_4$ measurements. The $\delta^{13}$C values of the CH$_4$ standard were not affected by combustion temperature at which CH$_4$ was oxidized to CO$_2$ between 765 and 840 °C. Subsequent gas samples thus were analyzed within this range of temperature. The $\delta^{13}$C values of a subset of CH$_4$ samples with concentrations ranging from 38 to 5279 μmol mol$^{-1}$ measured by TDLAS agreed well with those measured via isotope ratio mass spectrometry, with a slope of 0.97 and an R$^2$-value of 0.86 ($n = 32$). We independently analyzed a CH$_4$ standard (with a known value of -38.3‰) with [CH$_4$] diluted to 27, 55, and 150 μmol mol$^{-1}$, which measured -37.75 ± 2.73‰, -39.30 ± 1.39‰ and -39.14 ± 0.62‰ ($n = 9$ for each; one σ standard deviation), respectively. The $\delta^{13}$C values of the same standard diluted to 11 μmol mol$^{-1}$ were more variable, measuring -41.65 ± 5.23‰.
3.2. $\delta^{13}C$ values of CH$_4$ and CO$_2$

The Mollisol and Oxisol showed differing patterns in $\delta^{13}C$ values of CH$_4$ between the aerobic and anaerobic phases of the fluctuating treatment (Fig. 2a). Values of $\delta^{13}C$ of CH$_4$ were significantly more negative during the anaerobic phase (-43.97 ± 4.03‰) than the aerobic phase (-38.14 ± 5.69‰; $P < 0.01$) in the Mollisol. The $\delta^{13}C$ values of CH$_4$ in the Oxisol varied little between anaerobic and aerobic phases, averaging -49.16 ± 5.81‰ for the anaerobic phase and -48.06 ± 5.03‰ for the aerobic phase. The mean $\delta^{13}C$ values of CH$_4$ in the control were -38.14‰ for the Mollisol and -48.24‰ for the Oxisol.

The $\delta^{13}C$ values of CO$_2$ in the control were relatively stable over time (-10.13 ± 0.72‰ for the Mollisol and -14.90 ± 0.52‰ for the Oxisol), while $\delta^{13}C$ values of CO$_2$ varied with soil type and time in the fluctuating anaerobic/aerobic treatment (Fig. 2b). In the control, the Mollisol exhibited significantly higher $\delta^{13}C$ values of CO$_2$ than the Oxisol ($P < 0.01$). The $\delta^{13}C$ values of CO$_2$ in the fluctuating anaerobic/aerobic treatment were significantly more positive under the anaerobic phase (-9.05 ± 3.28‰ for the Mollisol and -8.81 ± 2.61‰ for the Oxisol) than the aerobic phase (-13.49 ± 1.18‰ for the Mollisol and -11.10 ± 2.53‰ for the Oxisol; $P < 0.01$ for both). The Mollisol showed a greater $\delta^{13}C$ value of CO$_2$ in the fluctuating anaerobic/aerobic treatment on the 4th day (1.91 ± 0.92‰). After that, the $\delta^{13}C$ values of CO$_2$ in the fluctuating anaerobic/aerobic treatment became similar to the control and gradually decreased over time ($P < 0.01$), with significantly lower values than the control after
44 days ($P < 0.05$). For the Oxisol, the $\delta^{13}C$ value of CO$_2$ in the fluctuating anaerobic/aerobic treatment increased after 24 days, and was significantly more positive than in the control ($P < 0.01$). Overall, at the end of the experiment, the fluctuating anaerobic/aerobic treatment exhibited a significantly lower cumulative $\delta^{13}C$ value of CO$_2$ in the Mollisol (-11.22 ± 0.43‰; $P < 0.05$) but a significantly higher value in the Oxisol (-10.83 ± 0.47‰; $P < 0.01$) relative to the controls (-9.85 ± 0.80‰ for the Mollisol and -14.89 ± 0.94‰ for the Oxisol).

The $\delta^{13}C$ values of total mineralized C was similar to $\delta^{13}C$ of CO$_2$ in the control, but not in the fluctuating anaerobic/aerobic treatment—especially for the Oxisol. The $\delta^{13}C$ values of total mineralized C in the control did not vary with time, and were significantly higher in the Mollisol (-10.60 ± 0.87‰) than in the Oxisol (-15.06 ± 0.95‰; $P < 0.01$). However, in the fluctuating anaerobic/aerobic treatment, the $\delta^{13}C$ values of total mineralized C in the Mollisol became much more negative.

3.3. $\delta^{13}C$ values of total mineralized C

The pattern of $\delta^{13}C$ values of total mineralized C was similar to $\delta^{13}C$ of CO$_2$ in the control, but not in the fluctuating anaerobic/aerobic treatment—especially for the Oxisol. The $\delta^{13}C$ values of total mineralized C in the control did not vary with time, and were significantly higher in the Mollisol (-10.60 ± 0.87‰) than in the Oxisol (-15.06 ± 0.95‰; $P < 0.01$). However, in the fluctuating anaerobic/aerobic treatment, the $\delta^{13}C$ values of total mineralized C in the Mollisol became much more negative.
relative to $\delta^{13}C$ of CO$_2$ between 12 and 44 days, resulting in the $\delta^{13}C$ values of total mineralized C fluctuating between -14.56‰ and -9.22‰ after 12 days. After 24 days, the $\delta^{13}C$ of total mineralized C in the Oxisol became lower in the fluctuating anaerobic/aerobic treatment than in the control, and fluctuated between -20.03‰ and -13.83‰ (Fig. 3a). After 90 days, the cumulative $\delta^{13}C$ value of total mineralized C in the Mollisol was significantly lower in the fluctuating anaerobic/aerobic treatment (-11.65 ± 0.30‰) than in the control (-10.36 ± 0.45‰; $P < 0.05$). In contrast to the cumulative $\delta^{13}C$ value of CO$_2$, the cumulative $\delta^{13}C$ value of total mineralized C in the Oxisol did not significantly differ between treatments (-15.67 ± 0.28‰ for the fluctuating anaerobic/aerobic treatment and -15.05 ± 0.72‰ for the control).

Values of $\varepsilon_{TC-CO_2}$, which reflected the impact of CH$_4$ on the $\delta^{13}C$ values of CO$_2$, strongly varied with time in the fluctuating anaerobic/aerobic treatment but not in the control (Fig. 3b). For the control, the change in $\delta^{13}C$ value of CO$_2$ relative to total mineralized C was small in both soils ($\varepsilon_{TC-CO_2} = -0.48 ± 0.30‰$ for the Mollisol and -0.16 ± 0.25‰ for the Oxisol) due to a low cumulative percentage of CH$_4$ to total C mineralization (1.84% for the Mollisol and 0.48% for the Oxisol). However, the $\delta^{13}C$ of total mineralized C strongly differed from that of CO$_2$ in the fluctuating anaerobic/aerobic treatment ($P < 0.01$), especially in the Oxisol. In the Mollisol, $\varepsilon_{TC-CO_2}$ was more negative under the anaerobic phase ($\varepsilon_{TC-CO_2} = -1.04 ± 1.25‰$) relative to the aerobic phase ($\varepsilon_{TC-CO_2} = -0.14 ± 0.17‰$) ($P < 0.01$). In the Oxisol, $\varepsilon_{TC-CO_2}$ became more negative after 24 days ($P < 0.01$), ranging from -12.00‰ under the anaerobic phase to -3.88‰ under the aerobic phase.
Variations in $\varepsilon_{TC-CO_2}$ were closely related to the percent contribution of CH$_4$ to C mineralization (defined here as CH$_4$ percentage; Fig. 3c). The CH$_4$ percentage in the control was always < 3.50% and <2.40% for the Mollisol and Oxisol, respectively, averaging 1.67% and 0.47%. The fluctuating anaerobic/aerobic treatment caused fluctuations in CH$_4$ percentage over time (Fig. 3c), especially in the Oxisol, due to relative changes in CO$_2$ and CH$_4$ production as a function of O$_2$ availability (Fig. 4). In the Mollisol, the fluctuating anaerobic/aerobic treatment produced higher CH$_4$ percentages between 12 and 44 days, ranging from 0.16% to 10.45% (mean of 3.25%), with significantly higher values during the anaerobic than the aerobic phases ($P < 0.01$). The fluctuating anaerobic/aerobic treatment in the Oxisol showed significantly greater CH$_4$ percentages than the control after 24 days ($P < 0.01$), ranging from 6.45% to 29.00% (mean of 18.22%).

To generalize our findings to other terrestrial mineral soils, we calculated the impacts of CH$_4$ percentage on $\varepsilon_{TC-CO_2}$ across a range of CH$_4$ production and $\varepsilon_{CO_2-CH_4}$ values corresponding with our data (Table 1). We showed three scenarios corresponding with minimum, mean, and maximum observed $\varepsilon_{CO_2-CH_4}$ values and contributions of CH$_4$ to total C mineralization from 2% to 50%. If CH$_4$ production was < 2% of total C mineralization, we would expect $\varepsilon_{TC-CO_2}$ of -0.96 – -0.28‰ based on an $\varepsilon_{CO_2-CH_4}$ between 14‰ and 48‰, respectively. The absolute magnitude of $\varepsilon_{TC-CO_2}$ values increased sharply as the percent of CH$_4$ increased: -2.40 – -0.70‰ given CH$_4$ production of 5%, and so on (Table 1).
4. Discussion

We found that terrestrial soils exposed to brief periods (4 days) of anaerobic conditions can produce small but significant CH₄ fluxes which continue to a lesser extent during subsequent periods of O₂ exposure. Consistent with our hypothesis, even the relatively small CH₄ fluxes observed here significantly altered the δ¹³C values of CO₂ (by as much as 12‰) relative to δ¹³C of total soil C mineralization, and thus critically altered their ecological interpretation. This is crucially important in the context of studies which aim to decipher the source of respiration using small (i.e., 1 – 4‰) differences in δ¹³C values associated with specific organic compounds (e.g., carbohydrates vs. lipids or lignin) or specific ecological sources (e.g., roots vs. microbes; Bowling et al., 2008). Many previous studies reporting measurements of δ¹³C of soil respiration have been conducted in periodically wet, C-rich soils from terrestrial or ephemeral wetland ecosystems where small net CH₄ fluxes may have been present (i.e., conditions similar to our study). These include studies from boreal peatlands and arctic tundra where relatively small differences in δ¹³C of CO₂ (several ‰) were interpreted in the context of identifying C substrates for CO₂ production (Dioumaeva et al., 2002; Oelbermann et al., 2008) and quantifying δ¹³C values of ecosystem respiration (Natali et al., 2011; Hicks Pries et al., 2013), but where CH₄ was not apparently measured. Our study suggests the possibility that some of this reported isotopic variation, and the otherwise intriguing differences between δ¹³C values of CO₂ and bulk soil δ¹³C, might derive from CH₄ production. In ecosystems where even small net positive CH₄ fluxes to the atmosphere occur (i.e.,
accounting for ~5% of total C mineralization), explicit consideration of CH₄ contributions to δ¹³C mass balance appears necessary for robust interpretation of δ¹³C of soil respiration.

4.1. Effects of dynamic redox environments on δ¹³C values of CH₄ and CO₂

Few studies have conducted frequent measurements of δ¹³C values of CO₂ during prolonged soil incubations (Breecker et al., 2015; Huang and Hall, 2017), and even fewer have measured δ¹³C values of both CO₂ and CH₄ (Conrad and Claus, 2009). The relatively higher δ¹³C values of CO₂ and CH₄ in the Mollisol than the Oxisol likely reflected their initial δ¹³C values of SOC (-22.0‰ for the Mollisol, a mixed C₃-C₄ agroecosystem, and -28.4‰ for the Oxisol, a C₃ forest).

However, the temporal trends in the δ¹³C values of CO₂ and CH₄ clearly reflected CH₄-driven C isotope fractionation under the fluctuating anaerobic/aerobic treatment. Under aerobic conditions, heterotrophic microbial respiration does not normally fractionate δ¹³C values to a major extent (Ehleringer et al., 2000; Breecker et al., 2015). Accordingly, CO₂ produced in the control likely reflected the original δ¹³C signature of soil organic C compounds from which CO₂ was derived. The relatively positive δ¹³C value of CO₂ (-10.13‰) in the Mollisol relative to C₄ litter (-13.3‰) and soil organic C (-22.0‰) may be attributed to a small proportion of CO₂ generated by the dissolution of carbonate (-2.08‰). In comparison, the more positive δ¹³C values of CO₂ under the anaerobic phase (-9.05‰ for the Mollisol and -8.81‰ for the Oxisol) coincided with higher CH₄ percentages, as is commonly observed in
consistently saturated wetland systems with much greater CH4 production (Corbett et al., 2013; Berger et al., 2018). Compared with the relatively stable δ13C value of CO2 in the control, the mean δ13C values of CH4 under the anaerobic phase (-43.97‰ for the Mollisol and -49.16‰ for the Oxisol) were consistent with a mixture of hydrogenotrophic and acetoclastic methanogenesis (Blaser and Conrad, 2016). When CH4 was produced under the anaerobic phase, the residual CO2 was enriched in 13C in order to balance the depleted 13C of CH4 (Fig. 1), leading to higher εCO2–CH4. On the other hand, CH4 oxidation under the aerobic phase subsequently increased δ13C values of CH4 and thus decreased εCO2–CH4 (Fig. 1). Relative to the Oxisol, the larger variations in δ13C values of CH4 and εCO2–CH4 between the anaerobic and aerobic phases in the Mollisol suggested that CH4 oxidation may have been more important. The mean difference in δ13C values of CH4 between the end of aerobic and anaerobic phases in the Mollisol (~6‰) was consistent with fractionation from CH4 oxidation, within the range of typical values (< 10‰; Whiticar, 1999). Thus, fluctuations in the δ13C values of CO2 and CH4 and εCO2–CH4 were determined by the combination of the contribution of methanogenesis to total C mineralization and CH4 oxidation, as well as any potential shifts in C sources accompanying dynamic redox conditions (Huang and Hall, 2017).

In addition, dissolution of a small fraction of the isotopically heavy carbonate in the Mollisol likely contributed to a one-time pulse of CO2 with very high δ13C values at day 4 accompanying decreased pH under the anaerobic phase. Temporary decreases in pH in alkaline soils are commonly observed following the onset of anaerobic
conditions due to the transient accumulation of organic acids (Kirk, 2004).

Finally, the $\delta^{13}C$ values of CH$_4$ measured by our TDLAS method agreed well with conventional isotope ratio mass spectrometry (IRMS). The precision for continuous-flow IRMS can be < 0.2‰ (Yarnes, 2013), which is slightly better than our method. However, the TDLAS method provided an alternative to IRMS for $\delta^{13}C$ value of CH$_4$ measurement with high throughout (20 samples h$^{-1}$) at much lower operating costs, which facilitated the high-frequency use of C isotopes to understand the highly dynamic C mineralization in soils with O$_2$ fluctuations.

4.2. Impacts of CH$_4$ flux on $\delta^{13}C$ values of soil respiration

Total mineralized C had lower $\delta^{13}C$ values than CO$_2$ under fluctuating anaerobic/aerobic conditions, suggesting that the $\delta^{13}C$ values of CO$_2$ did not directly reflect the sources of mineralized C in soils that produced CH$_4$. Similarly, previous work showed that $\delta^{13}C$ values of CO$_2$ became more positive with increasing CH$_4$/CO$_2$ ratios (Hodgkins et al., 2014). A previous study (Holmes et al., 2015) showed that after incubating peat under anaerobic conditions for 48 days, CH$_4$ production representing 2% and 9% of total mineralized C resulted in $\epsilon_{TC-CO_2}$ values of -3.2‰ and -8.3‰, respectively, due to the dominance of hydrogenotrophic methanogenesis with very high enrichment factors (> 80‰). In the mineral soils examined here, the $\epsilon_{CO_2-CH_4}$ values were smaller, suggesting that the influence of CH$_4$ fractionation on $\delta^{13}C$ values of CO$_2$ could potentially be neglected if CH$_4$ accounts for < 2%. This is consistent with our previous study examining CO$_2$ and CH$_4$ dynamics from another
Mollisol (Huang and Hall, 2017). In contrast, δ¹³C values of soil respiration were strongly affected (εTC–CO₂ up to -12‰) by the anaerobic/aerobic phase with CH₄ > 5% of total mineralized C, which supported our hypothesis. Thus, both the CH₄ percentages and εCO₂–CH₄ values are critical controls on δ¹³C values of soil respiration.

4.3. Implications for ecosystem δ¹³C dynamics

Net CH₄ production is traditionally thought to occur only after prolonged anaerobic conditions (Conrad, 1996), but our results showed that CH₄ production was highly significant even under short-duration O₂ fluctuations. This is consistent with previous reports of sporadic net CH₄ production from bulk aerobic terrestrial soils (Silver et al., 1999; von Fischer and Hedin, 2007; Liptzin et al., 2011; Hall et al., 2013). Similar to our study, net CH₄ production was also observed for several days after saturated soils became fully aerated (Ebrahimi and Or, 2017). Thus, CH₄ fractionation effects on δ¹³C values of soil respiration should not necessarily be ignored even in bulk aerobic soils. For example, Hicks Pries (2013) found that the δ¹³C value of respired CO₂ increased from ~ -26‰ to ~ -20‰ with depth (0 – 80 cm) in peatland soils incubated under aerobic conditions. In that study, the 6‰ increase in δ¹³C value of CO₂ was interpreted as a shift in C sources from plant to microbial-derived C with depth, even as bulk soil δ¹³C values varied little with depth (Hicks Pries et al., 2012). Our data suggest that a small increase in net CH₄ production with depth could also explain the observed increase in δ¹³C value of CO₂ and the 4.8‰ difference between δ¹³C values of CO₂ and bulk soil C in that study.
5. Conclusions

The results from this incubation experiment emphasized the necessity to have frequent measurements of CO₂ and CH₄ production and their δ¹³C values in soils that experienced O₂ fluctuations, in order to better reflect the δ¹³C values of total mineralized C. We found that short-duration fluctuations in O₂ availability produced small but significant CH₄ fluxes in the two mineral soils examined here, which strongly altered the δ¹³C values of CO₂. We observed smaller differences in δ¹³C values between CO₂ and CH₄ (14 – 48‰) than in previous studies of consistently saturated wetland soils. However, as hypothesized, when CH₄ represented > 5% of total soil C mineralization, consideration of δ¹³C values of CH₄ was necessary for robust interpretation of δ¹³C values of soil respiration in these dynamic redox environments.

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Table 1 Differences in δ^{13}C values between total mineralized C and CO₂ (ε_{TC-CO₂}) as a function of the percentage of CH₄ to total mineralized C (P_{CH₄}).

ε_{TC-CO₂} = \frac{-P_{CH₄}}{100} \times ε_{CO₂-CH₄}, where ε_{CO₂-CH₄} is C isotope separation between CO₂ and CH₄. The values of ε_{min}, ε_{mean} and ε_{max} were representative of our data. ε_{min}, minimum of ε_{CO₂-CH₄}; ε_{mean}, mean of ε_{CO₂-CH₄}; ε_{max}, maximum of ε_{CO₂-CH₄}.

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**Fig. 1** Schematic of processes affecting C isotope ratios ($\delta^{13}C$ values) of CH$_4$ and CO$_2$ in fluctuating anaerobic/aerobic soils. The numbers on the lines indicate C isotope enrichment factors ($\varepsilon$). The different letters on the lines indicate different processes: Production of CO$_2$ from soil respiration (a, b); soil organic C fermentation to acetate (c); acetoclastic methanogenesis (d); hydrogenotrophic methanogenesis (e); CH$_4$ oxidation (f). The solid line indicates aerobic conditions, and the dashed line indicates anaerobic conditions. The lines in black denote minor C fractionation; the lines in orange indicate that fractionation increased $\delta^{13}C$ values of CO$_2$; and the line in blue indicates that fractionation decreased $\delta^{13}C$ values of CO$_2$.

**Fig. 2** Carbon isotopes ($\delta^{13}C$ values) of CH$_4$ (a), CO$_2$ (b), and their difference ($\varepsilon_{CO_2-CH_4}$) (c) in the Mollisol and Oxisol under a fluctuating anaerobic/aerobic treatment and a static aerobic condition (control). $\varepsilon_{CO_2-CH_4} = \delta^{13}C_{CO_2} - \delta^{13}C_{CH_4}$. The solid line indicates aerobic conditions, and the dashed line indicates anaerobic conditions. The error bars indicate SD (n = 3 for each treatment).

**Fig. 3** Carbon isotope ($\delta^{13}C$ values) of total mineralized C (CH$_4$ and CO$_2$) (a), the difference in $\delta^{13}C$ values between total mineralized C and CO$_2$ ($\varepsilon_{TC-CO_2}$) (b), and CH$_4$ percentage contribution to total mineralized C (c) in the Mollisol and Oxisol under a fluctuating anaerobic/aerobic treatment and a static aerobic condition (control). The solid line indicates aerobic conditions, and the dashed line indicates anaerobic
conditions. The error bars indicate SD (n = 3 for each treatment).

**Fig. 4** Production rate of CH$_4$ and CO$_2$ in the Mollisol and Oxisol under a fluctuating anaerobic/aerobic treatment and a static aerobic condition (control). The solid line indicates aerobic conditions, and the dashed line indicates anaerobic conditions. The error bars indicate SD (n = 3 for each treatment).
Fig. 1
Fig. 2
Fig. 3
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