The stable carbon isotope signature of methane produced by saprotrophic fungi

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Received: 25 March 2020 – Discussion started: 2 April 2020
Revised: 9 June 2020 – Accepted: 24 June 2020 – Published: 31 July 2020

Abstract. Methane (CH4) is the most abundant organic compound in the atmosphere and is emitted from many biotic and abiotic sources. Recent studies have shown that CH4 production occurs under aerobic conditions in eukaryotes, such as plants, animals, algae, and saprotrophic fungi. Saprotrophic fungi play an important role in nutrient recycling in terrestrial ecosystems via the decomposition of plant litter. Although CH4 production by saprotrophic fungi has been reported, no data on the stable carbon isotope values of the emitted CH4 (δ13C-CH4 values) are currently available. In this study, we measured the δ13C values of CH4 and carbon dioxide (δ13C-CO2 values) emitted by two saprotrophic fungi, Pleurotus sapidus (oyster mushroom) and Laetiporus sulphureus (sulphur shelf), cultivated on three different substrates, pine wood (Pinus sylvestris), grass (mixture of Lolium perenne, Poa pratensis, and Festuca rubra), and corn (Zea mays), which reflect both C3 and C4 plants with distinguished bulk δ13C values. Applying Keeling plots, we found that the δ13C source values of CH4 emitted from fungi cover a wide range from −40 to −69 mUr depending on the growth substrate and fungal species. Whilst little apparent carbon isotopic fractionation (in the range from −0.3 to 4.6 mUr) was calculated for the δ13C values of CO2 released from P. sapidus and L. sulphureus relative to the bulk δ13C values of the growth substrates, much larger carbon isotopic fractionations (ranging from −22 to −42 mUr) were observed for the formation of CH4. Although the two fungal species showed similar δ13CH4 source values when grown on pine wood, δ13CH4 source values differed substantially between the two fungal species when they were grown on grass or corn. We found that the source values of δ13CH4 emitted by saprotrophic fungi are highly dependent on the fungal species and the metabolized substrate. The source values of δ13CH4 cover a broad range and overlap with values reported for methanogenic archaea, the thermogenic degradation of organic matter, and other eukaryotes.

1 Introduction

Methane (CH4) is an important greenhouse gas that is emitted by several abiotic sources (e.g. fossil fuel, biomass burning, and geological processes) and biotic sources (e.g. wetlands, agriculture and waste, and fresh waters) to the atmosphere (Kirschke et al., 2013; Saunois et al., 2016, 2020). In the past, biotic CH4 production has been attributed exclusively to strictly anaerobic microorganisms, such as methanogens, that are ubiquitous in wetlands, rice paddies, landfills, and the intestines of termites and ruminants (Kirschke et al., 2013). The discovery of CH4 emissions from dead and living plants under oxic conditions (Keppler et al., 2006, 2009) paved the way for the search for new biogenic CH4 sources. Since then, several previously unknown CH4 sources have been discovered, including the endothelial cells
of rat liver (Boros and Kepper, 2019; Ghyczy et al., 2008), plant cell cultures (Wishkerman et al., 2011), marine algae (Klintzsch et al., 2019; Lenhart et al., 2016), marine and terrestrial cyanobacteria (Biži´c et al., 2020), humans (Kepper et al., 2016), and saprotrophic fungi (Lenhart et al., 2012).

Fungi play a central role in ecosystems by decomposing organic matter, thereby recycling formerly bound carbon and nutrients (Grinshut et al., 2007). This process is especially important in forests where fungi are essential for wood decay and have a great impact on the carbon and nitrogen cycles (Ralph and Catcheside, 2002). White rot fungi (e.g. Trametes versicolor or Pleurotus ostreatus) are able to decompose the chemically complex structural component lignin, whereas brown rot fungi (e.g. Serpula lacrymans or Gloeocephalum trabeum) mainly metabolize cellulose and hemicellulose (Ten Have and Teunissen, 2001; Leonowicz et al., 1999; Valašková and Baldrian, 2006). Fungi have already been determined to be involved in the synthesis of CH₄ during wood decay (Beckmann et al., 2011; Mukhin and Voronin, 2007, 2008) via the breakdown of large macromolecules to smaller molecules, thereby providing bacteria and archaea with essential substrate. Elevated levels of CH₄ were found in fungus-infected wood stems with oxygen concentrations ranging from 1% to 14% (Hietala et al., 2015). Here, CH₄ production was associated with anoxic microsites in the xylem, indicating that at least part of the CH₄ was produced by methanogenic archaea. Nevertheless, Lenhart et al., 2012 demonstrated that basidiomycetes are able to produce CH₄ under aerobic conditions without the presence of methanogenic archaea. Therefore, fungi might be an underestimated source of CH₄ in the global CH₄ cycle.

Stable carbon isotopes (expressed as δ¹³C values) have often been used to investigate sources and sinks of CH₄ on the global scale (Whiticar, 1993). As different CH₄ sources have characteristic δ¹³C values, δ¹³C-CH₄ values might be used to quantify the individual contributions of various sources regionally and/or globally (Dlugokencky et al., 2011; Hein et al., 1997; Nisbet et al., 2016; Quay et al., 1999; Tyler, 1986; Whiticar, 1999). The short lifetime of CH₄ in the atmosphere (from 9.7 ± 1.5 to 11.2 ± 1.3 years; Naik et al., 2013; Prather et al., 2012; Voulgarakis et al., 2013) assures that global isotopic δ¹³C-CH₄ patterns represent the average of recent inputs by various sources and allows for the quantification of respective source strengths (Mikaloff Fletcher et al., 2004a, b).

Additionally, stable isotopes provide information about the formation processes of CH₄. Traditionally, three formation categories of δ¹³C-CH₄ values have been identified: biogenic, with typical δ¹³C-CH₄ values ranging from ~ −55 to −70 mUr; thermogenic, with typical δ¹³C-CH₄ values ranging from ~ −25 to −55 mUr; and pyrogenic, with typical δ¹³C-CH₄ values ranging from ~ −13 to −25 mUr (Kirschke et al., 2013). However, stable isotope values of recently identified CH₄ sources, i.e. human CH₄ emissions (~−56 to −95 mUr; Kepper et al., 2016), plant-derived CH₄ (~−52 to −69 mUr; Kepper et al., 2006), and abiotic UV-induced CH₄ formation by plants (~−52 to −67 mUr; Vigano et al., 2009) also need to be considered.

In this study, we investigated the stable carbon isotope source signatures of CH₄ and CO₂ released by the two basidiomycetes Pleurotus sapidus (white rot fungus) and Laetiporus sulphureus (brown rot fungus). Both fungi were cultivated under sterile conditions on three different substrates (pine wood, grass, and corn) with varying bulk δ¹³C values. We examined the influence of fungal species and growth substrate on δ¹³C-CH₄ and δ¹³C-CO₂ values and compared the δ¹³C-CH₄ values from the two fungal species with those of other known sources reported in the literature.

2 Material and methods

2.1 Selected fungi

P. sapidus (Pleurotaceae, DSMZ 8266) and L. sulphureus (Polyporaceae, DSMZ 1014) were chosen for this experiment because of their capability to emit CH₄ (Lenhart et al., 2012), their ecological and physiological characteristics (P. sapidus is a white rot fungus and L. sulphureus is a brown rot fungus), and their well-established practical handling under laboratory conditions.

2.2 Cultivation of fungi and incubation experiments

Pine wood (Pinus sylvestris), grass (mixture of Lolium perenne, Poa pratensis, and Festuca rubra) and corn (Zea mays) were selected as growth substrates. Pine wood was chosen to investigate if white rot and brown rot fungi differ with respect to the δ¹³C-CH₄ and δ¹³C-CO₂ values released during wood decay. Therefore, dead pine wood branches were collected from the forest floor and shredded to small wood chips with a length of about 5 cm (Natura 1800L; Gloria, Witten, Germany). The wood chips were dried at 60 °C for 48 h and stored in a flask (WECK GmbH, Hanau, Germany). Grass (C₃ plant) and corn (C₄ plant) were selected because of their different stable carbon values. As the metabolic pathway for carbon fixation is biochemically different in C₃ and C₄ plants, plant biomass differs with respect to δ¹³C values, which, in turn, might lead to different δ¹³C values of CH₄ and CO₂ released by fungi. Therefore, typical garden lawn was manually cut, dried at 70 °C, and stored in a flask. The corn substrate consisted of conventional corn flour.

The substrates were autoclaved, filled into 2.7 L flasks (WECK GmbH, Hanau, Germany), and inoculated with pure fungal submerged cultures under sterile conditions following Lenhart et al. (2012). After the addition of the fungi, the flasks were closed with lids and a rubber band sealing. To allow gas exchange during the fungal growth period (about 2 weeks), a hole in the centre of every lid was fitted with a cotton stopper. Before the start of the incubation experi-
ments, the flasks were aerated under sterile conditions in or-
der to start the incubation at atmospheric CH$_4$ mixing ratios. 
Additionally, to create an airtight seal, the flask’s cotton stop-
ners were replaced by sterile silicone stoppers (Saint-Gobain 
Performance Plastics, Charny, France).

For the incubation experiments, *P. sapidus* and *L. sul-
phureus* were incubated on the three substrates, while 
uninoculated substrates were also incubated as control treat-
ments. Before the incubation experiments, the substrates 
were sterilized by autoclaving at 121 °C and 2 bar pressure 
for 20 min. The incubation experiments were conducted us-
ing three replicates per treatment. The duration of the incuba-
tion accounted for up to 40 h. All incubations were conducted 
at room temperature (23 ± 1.5 °C). At every sampling point, 
40 mL of air was taken from the flasks for gas concentration 
measurements and an additional 40 mL was taken for δ$^{13}$C-
CH$_4$ stable isotope ratio mass spectrometry (IRMS) analysis. 
The gas samples were taken with airtight 60 mL PE syringes 
(Plastipak, BD, Franklin Lakes, USA) and transferred into 12 
ml evacuated Exetainer vials (Labco, High Wycombe, 
UK). Subsequently, a volume of atmospheric air equivalent 
to the volume of the removed sample was added into each 
flask directly after sampling. Mixing ratios and stable isotope 
values of CH$_4$ were corrected according to the dilution. 

When calculating the fungal CH$_4$ and CO$_2$ production 
rates, we subtracted substrate-derived CH$_4$ and CO$_2$ production 
rates (determined using the control treatments) from the 
respective fungi-containing samples. Additionally, only sam-
ple points showing a linear increase in CH$_4$ and CO$_2$ were 
considered in the calculation of the fungal production rates.

To account for differences in the metabolic activity of the 
fungi, we additionally measured respiration rates, assuming 
that metabolic activity correlates with respiration and, there-
fore, the CO$_2$ emissions of the fungi. Hence, we related 
fungus-derived CH$_4$ emissions to respiration by calculating 
the CH$_4$ : CO$_2$ emission ratio.

2.3 Analysis of CH$_4$ and CO$_2$ via gas chromatography

Samples were analysed using a gas chromatograph (GC, 
Bruker Greenhouse Gas Analyser 450-GC) equipped with 
a flame ionization detector (FID) and an electron capture 
detector (ECD) for the detection of CH$_4$ and CO$_2$ respec-
tively. The detector temperatures were set at 300 °C (FID) 
and 350 °C (ECD). Five reference gases (Deuste Steininger 
GmbH) were used to calibrate the GC system. The reference 
gases were in the range from 1 ppmv (parts per million by 
volume) to 21 and from 304 to 40 000 ppmv for CH$_4$ and 
CO$_2$ respectively. Gas peaks were integrated using Galaxie 
software (Varian Inc., Palo Alto, CA, USA).

2.4 Definition of δ values and isotope apparent 
fractionation

In this paper, all stable carbon isotope ratios are expressed 
in the conventional “delta” (δ) notation, meaning the relative 
difference of the isotope ratio of a substance from the stan-

δ$^{13}$C = \( \frac{^{13}C}{^{12}C} \) sample − 1 \tag{1}

The apparent fractionation (ε$_{app}$) between fungal δ$^{13}$C-CH$_4$ or δ$^{13}$C-CO$_2$ values and the δ$^{13}$C values of the substrates was calculated according to Eq. (2):

ε$_{app}$ CH$_4$ or CO$_2$ = \( \frac{\delta^{13}C + 1}_{\text{fungal CH}_4 \text{ or CO}_2} - 1 \) \tag{2}

We follow the proposal of Brand and Coplen (2012) and use the term “permil” (‰) as the isotope delta unit, in order to 
conform with the guidelines for the International System of 
Units (SI). Hence, isotope delta values that were formerly 
given as −70‰ are expressed as −70 mUr.

2.5 Measurements of δ$^{13}$CH$_4$ and δ$^{13}$CO$_2$ values

Stable carbon isotope values of CH$_4$ and CO$_2$ were measured 
using a continuous flow isotope mass spectrometry system 
(CF-IRMS). A HP 6890N GC (Agilent, Santa Clara, USA) 
was linked to a pre-concentration unit for CH$_4$ measure-
ments and an A200S autosampler (CTC Analytics, Zwingen, 
Switzerland) for CO$_2$ analysis. The GC was equipped with a 
CP-PoraPLOT Q capillary column (27.5 m x 0.25 mm i.d., 
film thickness 8 µm; Varian, Palo Alto, USA). The GC was 
operated with an injector temperature of 200 °C, an isother-
mal oven temperature of 30 °C, split injection (10 : 1), and a 
constant carrier gas flow of 1.8 mL min$^{-1}$ (methane-free 
helium). The GC was coupled to a DeltaPLUS XL isotope ratio 
mass spectrometer (ThermoQuest Finnigan, Bremen, Ger-
many) via an oxidation reactor and a GC Combustion III 
interface (ThermoQuest Finnigan, Bremen, Germany). The 
oxidation reactor was employed with the following proper-
ties: a ceramic tube (Al$_2$O$_3$; length 320 mm, 1.0 mm i.d.) 
with Ni/Pt wires inside activated by oxygen and a reactor 
temperature of 960 °C.

For CH$_4$ measurements with the pre-concentration unit, 
headspace gas samples were transferred to an evacuated 
40 mL sample loop. Methane was trapped on HayeSep D, 
separated from other compounds by the GC, and then intro-
duced into the IRMS system via an open split. The moni-
tor gas was carbon dioxide of high purity (carbon dioxide 
4.5, Messer Griesheim, Frankfurt, Germany) with a known 
δ$^{13}$C value of −23.6 mUr (calibrated at the Max Planck 
Institute for Biogeochemistry in Jena, Germany). All δ$^{13}$C val-
ues were corrected using two CH$_4$ reference standards (Isom-
metric instruments, Victoria, Canada) with δ$^{13}$C values of
M. Schroll et al.: The stable carbon isotope signature plots. Corrected $\delta^{13}$C-CH$_4$ source values for P. sapidus and L. sulphureus are given as the arithmetic mean of the three individual flasks per treatment with their standard deviations ($n = 3$).

The determination coefficient ($R^2$) of the Keeling plots showed values higher than 0.93 except for P. sapidus grown on grass ($R^2 = 0.51$). The lower $R^2$ value for P. sapidus grown on grass is probably a result of the marginal changes in the $\delta^{13}$C-CH$_4$ values due to the small increase in the CH$_4$ mixing ratio compared with the background CH$_4$ mixing ratio. Therefore, the low $R^2$ does not necessarily indicate a weaker relationship between the CH$_4$ mixing ratio and the $\delta^{13}$C-CH$_4$ value.

2.8 Statistics

Mixing ratios and production rates of CH$_4$, CO$_2$, $\delta^{13}$C-CH$_4$, values, and $\delta^{13}$C-CO$_2$ values as well as $\delta^{13}$C source values are presented as the arithmetic mean of three independent replicates with their standard deviations ($n = 3$). Linear regression analysis, the arithmetic means, and their standard deviations were calculated using Microsoft Excel (Microsoft Excel for Office 365 MSO). Two-way analysis of variance (ANOVA) tests (SigmaPlot 12.2.0.45, USA) were carried out to test for species- and substrate-related effects on the $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-CO$_2$ source values for each treatment. Differences at the $p < 0.05$ level were referred to as significant.

3 Results and discussion

In this section, we firstly present the results of CH$_4$ and CO$_2$ production from the two fungal species grown on the three different substrates. This includes emission rates of CH$_4$ and CO$_2$ from the control treatments of pine wood, grass, and corn as well as the molar ratio of CH$_4$ and CO$_2$. Secondly, we then present the respective stable isotope values measured for CH$_4$ and CO$_2$ during the incubation experiments and calculate the stable isotope source values of CH$_4$ and CO$_2$ released by the fungi applying Keeling plots. We then compare these values with stable carbon isotope values of the bulk organic matter by calculating the apparent fractionation. Finally, we compare $\delta^{13}$C source values of fungus-derived CH$_4$ with known values for other CH$_4$ sources from the literature.

3.1 Release of CH$_4$ and CO$_2$ from P. sapidus and L. sulphureus

All incubation experiments in which fungi were grown on different substrates showed a significant increase in CH$_4$ compared with the respective substrate control (Fig. 1a, c). Calculated emission rates for CH$_4$ and CO$_2$ are presented in Table 1. L. sulphureus grown on grass (7.5 ± 1.3 nmol h$^{-1}$) showed the highest emission rate of CH$_4$, followed by L. sulphureus grown on pine (6.2 ± 0.3 nmol h$^{-1}$), P. sapidus grown on corn (4.4 ± 1.9 nmol h$^{-1}$), L. sulphureus grown
CH are not related to fungal biomass. Therefore, differences in their CH reflects the amount of fungal biomass and is also an indicator

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Mixing ratios of CH

Figure 1. Mixing ratios of CH4 and CO2 of P. sapidus (a, c) and L. sulphureus (b, d) grown on pine wood, grass, and corn. Mixing ratios are presented as mean values with their standard deviation (n = 3).

on corn (2.6 ± 0.1 nmol h⁻¹), P. sapidus grown on pine (2.5 ± 0.2 nmol h⁻¹), and P. sapidus grown on grass (1.4 ± 0.5 nmol h⁻¹). Please note that CH4 and CO2 emission rates are not related to fungal biomass. Therefore, differences in the emission rates might be due to varying fungal biomass of the subsamples. Instead, CH4 production was related to CO2 production by determining the molar emission ratio between CH4 and CO2 (µmol CH4 : mol CO2). Thus, CO2 production reflects the amount of fungal biomass and is also an indicator of the metabolic activity of the fungi.

The control flasks did not show significant changes in their CH4 and CO2 mixing ratios over time, except for CH4 in pine wood controls (1.3 ± 0.1 nmol h⁻¹). However, small CH4 emission rates of 0.25 ± 0.01 nmol h⁻¹ were observed in the control flasks of corn, and the CH4 mixing ratio slightly decreased over time in the grass control (−0.05 ± 0.04 nmol h⁻¹). Whilst the pine wood and corn control flasks showed a small increase in the CH4 mixing ratio, they did not show an increase in the CO2 mixing ratios. These data rule out contamination by microbial heterotrophs, as this would cause a measurable CO2 increase within the flasks. The CH4 increase in the substrate controls might be attributed to CH4 release by dead plant material, as was already shown by Keppler et al. (2006) and Vigano et al. (2009). Within the scope of these experiments, no analytic test for microbial contamination was conducted. Nevertheless, using three different methods (fluorescence in situ hybridization, FISH; confocal laser scanning microscopy, CLSM; and quantitative real-time polymerase chain reaction), Lenhart et al. (2012) clearly showed that no methanogenic archaea were present in the same method of cultivation of fungi and incubation experiments that were performed in this study. Furthermore, CH4 and CO2 release and the CH4 : CO2 emission ratios in our incubations are similar to the experiments of Lenhart et al. (2012) and do not indicate microbial contamination.

Therefore, we assume that no contamination with bacteria or methanogenic archaea was present in our investigations.

For P. sapidus grown on corn and L. sulphureus grown on grass, no further linear increase in CH4 was observed after 22 and 10 h respectively. This might be due to the reduced decay of organic matter and slower fungal metabolism owing to higher CO2 and lower O2 mixing ratios.

A drastic increase in CO2 mixing ratios relative to the controls was observed in all flasks containing fungi (Fig. 1b, d). The CO2 emission rates are shown in Table 1. CO2 production rates ranged from 176 ± 4 to 2910 ± 410 µmol h⁻¹ for P. sapidus grown on grass and P. sapidus grown on corn respectively. These highly variable CO2 production rates might reflect different fungal biomass and metabolic activity (mineralization of organic matter). In the control treatments, tiny increases in the CO2 mixing ratio were detected ranging from 0.64 ± 0.12 to 0.91 ± 0.14 µmol h⁻¹. Only one flask (corn control) showed a somewhat higher increase in CO2 (7.76 µmol h⁻¹), which was most likely caused by microbial contamination of the flask. However, no increase in the CH4 mixing ratio was detected (see Supplement). Therefore, this control flask was excluded from further calculations.

Mean CH4 and CO2 emission rates and CH4 : CO2 emission ratios of all treatments are presented in Table 1. Higher ratios indicate a higher CH4 production during decay of the substrates. Therefore, both fungal species and substrate affect the CH4 : CO2 emission ratio (p < 0.001). For P. sapidus, CH4 : CO2 emission ratios are more variable (1.4 to 8.0 µmol CH4/mol CO2) compared with L. sulphureus (6.7–9.6 µmol CH4/mol CO2). This variation might be due to differences in the fungi’s enzyme sets required for organic matter decay, as P. sapidus is a white rot fungus and L. sulphureus is a brown rot fungus. At present, the biochemical pathways that lead to CH4 are still unknown, although compounds such as the sulfur-bound methyl-group of methionine and glucose have been identified to act as carbon precursors of fungus-derived CH4 (Lenhart et al., 2012).

Lenhart et al., 2012 found CH4 : CO2 ratios of fungi that ranged between 8 µmol CH4/mol CO2 and 17 µmol CH4/mol CO2, which is in the same order of magnitude as the CH4 : CO2 ratios determined in this study. It should be noted that, for better comparability, the CH4 : CO2 ratios of Lenhart et al. (2012; given in ppbv CH4 : % CO2) were converted to fit the CH4 : CO2 ratio units used in this study (µmol CH4 : mol CO2).

Table 1. CH₄ and CO₂ production rates and molar CH₄ : CO₂ emission ratios of the fungi incubated on different substrates. Values are presented as mean values of three independent replicates with their standard deviation (n = 3), except for the control “corn” (n = 2).

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Substrate</th>
<th>CH₄ production rate (nmol h⁻¹)</th>
<th>CO₂ production rate (µmol h⁻¹)</th>
<th>CH₄ : CO₂ ratio (µmol mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. sapidus</td>
<td>Pine</td>
<td>2.5 ± 0.2</td>
<td>901 ± 79</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>1.4 ± 0.5</td>
<td>176 ± 4</td>
<td>8.0 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>4.4 ± 1.9</td>
<td>2910 ± 419</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>L. sulphureus</td>
<td>Pine</td>
<td>6.2 ± 0.3</td>
<td>724 ± 42</td>
<td>8.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>7.5 ± 1.3</td>
<td>771 ± 103</td>
<td>9.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>2.6 ± 0.1</td>
<td>385 ± 20</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>Pine</td>
<td>1.3 ± 0.1</td>
<td>0.64 ± 0.12</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>−0.05 ± 0.04</td>
<td>0.91 ± 0.14</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>0.25</td>
<td>0.66</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 2. Stable carbon isotope values of CH₄ and CO₂ of P. sapidus (a, c) and L. sulphureus (b, d) grown on pine, grass, and corn. Values are presented as mean values with their standard deviation (n = 3), except for δ¹³CO₂ values of L. sulphureus grown on corn (n = 2).

3.2 Stable carbon isotope values of CH₄ and CO₂

Stable carbon isotope values of CH₄ and CO₂ measured from the incubation experiments are presented in Fig. 2. All incubations show a trend towards more negative δ¹³C-CH₄ values (less ¹³C) with time except for P. sapidus grown on corn, where a tendency towards more positive δ¹³C-CH₄ values was observed (Fig. 2a, b). During the incubation, δ¹³C-CH₄ values changed from −47.7 ± 0.1 mUr (for the incubation of P. sapidus grown on pine/grass) and −48.2 ± 0.1 mUr (for the incubation of P. sapidus grown on corn and L. sulphureus grown on pine/grass/corn) to −53.0 ± 0.7 mUr (for P. sapidus grown on pine), −48.7 ± 0.3 mUr (for P. sapidus grown on grass), −45.8 ± 1.2 mUr (for P. sapidus grown on corn), −55.1 ± 0.4 mUr (for L. sulphureus grown on pine), −55.4 ± 0.4 mUr (for L. sulphureus grown on grass), and −49.9 ± 0.4 mUr (for L. sulphureus grown on corn). The controls showed no significant shift in δ¹³C-CH₄ values except for the pine control, where an increase in the CH₄ mixing ratio along with more negative values of δ¹³C-CH₄ values occurred over time. This was accounted for when calculating the δ¹³C-CH₄ source signatures for P. sapidus grown on pine and L. sulphureus grown on pine (see Sect. 2.7).

The δ¹³C-CO₂ values showed a trend towards more negative values within the first 3–4 h of incubation (Fig. 2c, d). After this time, only minor changes in the δ¹³C-CO₂ values occurred. Final δ¹³C-CO₂ values of the incubation were −24.9 ± 0.6 mUr (for P. sapidus grown on pine), −28.6 ± 0.9 mUr (for P. sapidus grown on grass), −12.0 ± 0.3 mUr (for P. sapidus grown on corn), −24.1 ± 0.1 mUr (for L. sulphureus grown on pine), −27.7 ± 0.5 mUr (for L. sulphureus grown on grass), and −13.0 ± 0.5 mUr (for L. sulphureus grown on corn).

3.3 δ¹³C-CH₄ and δ¹³C-CO₂ source signatures of fungi

The δ¹³C-CH₄ source signatures determined via a Keeling plot analysis (Fig. 3) that ranged from −69.2 ± 1.9 mUr (for L. sulphureus grown on grass) to −39.8 ± 2.0 mUr (for P. sapidus grown on corn) are presented in Table 2. Average δ¹³C-CH₄ source signatures for each fungal species, considering all three substrates, are −52.6 mUr for P. sapidus and −61.3 mUr for L. sulphureus. These results suggest that the fungal species significantly influence the isotopic values of the emitted CH₄ (p < 0.001). A possible explanation for this observation could be the different enzyme sets of both fungi that decompose different components of the growth substrates, as P. sapidus belongs to white rot fungi and L.
Table 2. Calculated $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-CO$_2$ source signatures, $\delta^{13}$C values of the substrates, and $\varepsilon_{app}$ CH$_4$ and $\varepsilon_{app}$ CO$_2$. Values are presented as mean values with their standard deviation ($n = 3$).

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Substrate</th>
<th>$\delta^{13}$C-CH$_4$ source (mUr)</th>
<th>$\delta^{13}$C-CO$_2$ source (mUr)</th>
<th>$\delta^{13}$C substrate (mUr)</th>
<th>$\varepsilon_{app}$ CH$_4$ (mUr)</th>
<th>$\varepsilon_{app}$ CO$_2$ (mUr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. sapidus</td>
<td>Pine</td>
<td>$-65.3 \pm 1.1$</td>
<td>$-24.1 \pm 0.1$</td>
<td>$-38.4 \pm 1.2$</td>
<td>$4.0 \pm 0.1$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>$-52.9 \pm 1.6$</td>
<td>$-27.4 \pm 1.3$</td>
<td>$-21.8 \pm 1.7$</td>
<td>$4.6 \pm 1.3$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>$-39.8 \pm 2.0$</td>
<td>$-12.0 \pm 0.3$</td>
<td>$-28.5 \pm 2.0$</td>
<td>$-0.3 \pm 0.3$</td>
<td></td>
</tr>
<tr>
<td>L. sulphureus</td>
<td>Pine</td>
<td>$-61.4 \pm 0.5$</td>
<td>$-25.0 \pm 0.5$</td>
<td>$-34.4 \pm 0.6$</td>
<td>$3.0 \pm 0.4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td>$-69.2 \pm 1.9$</td>
<td>$-29.0 \pm 0.5$</td>
<td>$-38.6 \pm 2.0$</td>
<td>$2.9 \pm 0.5$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>$-53.4 \pm 1.1$</td>
<td>$-12.8 \pm 0.3$</td>
<td>$-42.2 \pm 1.1$</td>
<td>$-1.1 \pm 0.3$</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Pine</td>
<td></td>
<td></td>
<td>$-28.0 \pm 0.5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grass</td>
<td></td>
<td></td>
<td>$-31.5 \pm 0.6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td></td>
<td></td>
<td>$-11.7 \pm 1.1$</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 3. Keeling plots shown for P. sapidus (a) and L. sulphureus (b) grown on the three substrates. Sample points in the graphs are given as the arithmetic mean of the $\delta^{13}$C-CH$_4$ or $\delta^{13}$C-CO$_2$ values with their standard deviation ($n = 3$) on the $y$ axis and the arithmetic mean of the inverted mixing ratio of CH$_4$ or CO$_2$ with their standard deviation ($n = 3$) on the $x$ axis.

Figure 4. Calculated source signatures of $\delta^{13}$C-CH$_4$ values (a) and $\delta^{13}$C-CO$_2$ values (b) from P. sapidus, L. sulphureus as well as the $\delta^{13}$C values of the substrate. The data points represent the mean values of the individual Keeling plots with their standard deviation ($n = 3$).

L. sulphureus is a brown rot fungus. However, detailed investigations of the metabolic pathways leading to CH$_4$ formation were beyond the scope of this study.

Furthermore, a significant effect of the growth substrate on $\delta^{13}$C-CH$_4$ source signatures was observed ($p < 0.001$). $\delta^{13}$C-CH$_4$ source signatures from P. sapidus were more positive compared with those of L. sulphureus when grown on grass ($\Delta = 16.3$ mUr) and corn ($\Delta = 13.6$ mUr) (Fig. 4). When grown on pine wood, $\delta^{13}$C-CH$_4$ source signatures were similar, with P. sapidus showing slightly more negative values ($\Delta = -3.9$ mUr). Methane emitted by both fungi grown on corn was generally more enriched in $^{13}$C (less negative $\delta^{13}$C-CH$_4$ source values) compared with the fungi grown on pine wood and grass. This might be easily explained by the $\delta^{13}$C values of the corn growth substrates ($-11.7$ mUr, which is typical for C$_4$ plants) being roughly 20 mUr less negative in their $\delta^{13}$C values compared with the C$_3$ plants, pine wood ($-28.0$ mUr) and grass ($-31.5$ mUr).

The comparison of calculated $\delta^{13}$C-CH$_4$ source signatures with measured bulk $\delta^{13}$C values of the substrates shows that CH$_4$ emitted by both fungi is generally depleted in $^{13}$C compared with the respective substrates (Fig. 4a). Based on these data, we further calculated the apparent fractionation ($\varepsilon_{app}$ CH$_4$) between the $\delta^{13}$C-CH$_4$ source signatures and the bulk $\delta^{13}$C values of the growth substrates. The apparent fractionation was calculated as no metabolic pathway for the formation of CH$_4$ in fungi is presently known; therefore, only the initial $\delta^{13}$C signatures of the substrates and the calculated $\delta^{13}$C-CH$_4$ source signatures of the fungi can currently be compared. The values of $\varepsilon_{app}$ CH$_4$ that range from $-21.8$ mUr (for P. sapidus grown on grass) to $-42.2$ mUr (for L. sulphureus grown on corn) are presented in Table 2. When grown on pine wood, $\varepsilon_{app}$ CH$_4$ values are similar for P. sapidus ($-38.4 \pm 1.2$ mUr) and L. sulphureus ($-34.4 \pm 0.6$ mUr). The differences in $\varepsilon_{app}$ CH$_4$ values between both fungal species are distinct when grown on grass (P. sapidus: $-21.8 \pm 1.7$ mUr; L. sulphureus: $-38.6 \pm$
The calculated $\delta^{13}$C-CO$_2$ source signatures of both fungi (Table 2) range from $-29.0 \pm 0.5$ mUr (for L. sulphureus grown on grass) to $-12.0 \pm 0.3$ mUr (for P. sapidus grown on corn). $\delta^{13}$C-CO$_2$ source signatures are in a similar range for both fungi for all three substrates. However, CO$_2$ emitted by L. sulphureus is slightly more depleted in $^{13}$C for all three substrates compared with P. sapidus. Hence, the effect of fungal species on the stable carbon isotope values of CO$_2$ is significant ($p = 0.008$). Moreover, the substrates used were found to influence $\delta^{13}$C-CO$_2$ values significantly ($p < 0.001$).

The $\delta^{13}$C-CO$_2$ source signatures of the fungi show only small deviations from the bulk $\delta^{13}$C values of the respective substrates (Fig. 4b). However, for both fungi grown on pine wood and grass, $\delta^{13}$C-CO$_2$ values are slightly less negative (a few mUr) compared with the bulk substrate. This observation is rather unexpected, as $\delta^{13}$C-CO$_2$ values are usually more negative with respect to $\delta^{13}$C values of growth substrates due to fractionation during metabolism (Bowling et al., 2008). However, when grown on corn $\delta^{13}$C-CO$_2$ source signatures from both fungi are more negative compared with the substrate, and calculated $\varepsilon_{\text{app}}$CO$_2$ values (Table 2) are $-1.1 \pm 0.3$ mUr and $+4.6 \pm 1.3$ mUr for L. sulphureus grown on corn and P. sapidus grown on grass respectively.

The results of the incubation experiments show that there are distinct differences in the $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-CO$_2$ values released by both fungi. While the $\delta^{13}$C-CO$_2$ source signatures are similar to the $\delta^{13}$C values of the substrate (with $\varepsilon_{\text{app}}$CO$_2$ values of up to 4.6 mUr), the $\delta^{13}$C-CH$_4$ source signatures deviate strongly from the respective substrate, with $\varepsilon_{\text{app}}$CH$_4$ values of up to $-42.2$ mUr. This either indicates that metabolic pathways leading to the formation of CH$_4$ and CO$_2$ have different fractionation and/or that fungal CH$_4$ and CO$_2$ are derived from different precursor compounds of the respective substrate. The growth substrates used for this study (pine wood, grass, and corn) contain distinct amounts of cellulose, hemicellulose, lignin, and other compounds in different proportions (in contrast to only using pure glucose or cellulose as the growth substrate). Hence, the $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-CO$_2$ source signatures depend on the specific metabolic pathways used by the fungal species as well as the chemical composition of the growth substrate. The selected fungi and the growth substrates used provide a first solid basis for the potential range of $\delta^{13}$C-CH$_4$ values that might occur in nature.

### 3.4 Fungal $\delta^{13}$C-CH$_4$ values compared with known CH$_4$ sources

Figure 5 compares the $\delta^{13}$C-CH$_4$ values emitted by fungi in relation to other known CH$_4$ sources in the environment that have been reported in the literature. The red bars indicate typical biogenic (formerly only considered to be produced by archaea) CH$_4$ sources with emissions from wetlands, ruminants, landfills, and rice paddies where $\delta^{13}$C-CH$_4$ values usually range from $-85$ to $-40$ mUr. Abiotic CH$_4$ sources (including thermogenic or pyrolytic processes) stemming from natural gas, coal mining, and biomass burning are characterized by less negative $\delta^{13}$C values usually ranging from $-55$ to $-20$ mUr. In addition, gas hydrates, which might be formed by both microbial and abiotic processes, cover a wide range of $\delta^{13}$C values ($-29$ to $-73$ mUr) depending on their formation mechanisms (Kvenvolden, 1995). The $\delta^{13}$C source signatures of plant-derived CH$_4$ have been reported to be in the range of $-72$ to $-45$ mUr (Keppler et al., 2006; Vigano et al., 2009) depending on their photosynthetic pathways (C$_3$, C$_4$, or CAM). Furthermore, there was a tendency towards more negative $\delta^{13}$C-CH$_4$ values when the respective plant was treated with UV radiation (Vigano et al., 2009). $\delta^{13}$C-CH$_4$ source signatures of humans, which might include formation by microbes in the gut as well as formation by cellular processes, show a rather wide range with values between $-95$ and $-56$ mUr (Keppler et al., 2016). The results of our experiments conducted with two fungal species and three different growth substrates provide a range of $\delta^{13}$C-CH$_4$ source values from $-69$ to $-40$ mUr. This range overlaps with other eukaryotic sources, most microbial CH$_4$ sources, and even some abiotic CH$_4$ sources such as natural gas or emissions from coal mining.

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![Figure 5](https://i.imgur.com/5.png)

**Figure 5.** The range of $\delta^{13}$C-CH$_4$ values of microbial CH$_4$ sources (red), abiotic CH$_4$ sources (grey), eukaryotic CH$_4$ sources (green), atmospheric CH$_4$ (blue), and fungal CH$_4$ from this study (orange). The red and grey dashed bar indicates a mixture of microbial and abiotic CH$_4$ formation processes for gas hydrates (Kvenvolden, 1995). Data were taken from Brownlow et al. (2017), Keppler et al. (2006, 2016), Kvenvolden (1995), Nisbet et al. (2016), Quay et al. (1999), and Vigano et al. (2009).
4 Conclusions

This study provided the first analysis of stable carbon isotope values of \( \text{CH}_4 \) emitted by two saprotrophic fungi that were grown on three different substrates. \( \delta^{13}\text{C}-\text{CH}_4 \) and \( \delta^{13}\text{C}-\text{CO}_2 \) source values were found to be dependent on the fungal species as well as the substrates decomposed by the fungi. \( \delta^{13}\text{C}-\text{CH}_4 \) source values of the fungi were found to be in the range of \(-69\) to \(-40\) mUr and, therefore, overlap with \( \delta^{13}\text{C}-\text{CH}_4 \) values reported for other \( \text{CH}_4 \) sources such as methanogenic archaea, eukaryotes, and from abiotic \( \text{CH}_4 \) sources (e.g. natural gas and coal mining). Stable carbon isotope values of \( \text{CH}_4 \) in combination with flux measurements are often applied for a better understanding of regional and global \( \text{CH}_4 \) cycling. However, in recent years it has become clear that many biogenic \( \text{CH}_4 \) sources include complex \( \text{CH}_4 \) formation processes, resulting in different isotopic fractionation patterns depending on several biochemical and abiotic factors. Thus, studying ecosystems in which more than one major \( \text{CH}_4 \) source has to be expected (e.g. methanogenic archaea, fungi, cyanobacteria, or plants) becomes increasingly complicated, as distinguishing between each individual source based solely on stable carbon isotope values might be highly challenging. Therefore, additional tools are needed to better identify the sources but also to disentangle sources and sinks. In future research, stable hydrogen isotopic values of \( \text{CH}_4 (\delta^{2}\text{H}-\text{CH}_4 \text{ values}) \) or even applications of clumped isotopes might prove to be suitable tools to better distinguish between different \( \text{CH}_4 \) sources and, thus, to better constrain the global \( \text{CH}_4 \) budget.

Data availability. The data used in this study are available from heiDATA, which is an institutional repository for research data of the Heidelberg University (https://doi.org/10.11588/data/DQYPMC, Schroll et al., 2020).

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/bg-17-3891-2020-supplement.

Author contributions. MS, KL, and FK conceived the study and designed the experiments. HZ provided fungal cultures. MS performed the experiments under the supervision of FK and KL. CE helped with gas measurements. MG measured stable isotope values of greenhouse gases. MS, FK, and KL analysed the data. MS, FK, HZ, MG, and KL discussed the results, and MS, KL and FK wrote the paper.

Competing interests. The authors declare that they have no conflict of interest.

Acknowledgements. We thank Anette Giesemann for analytical measurements of stable carbon isotope values of the bulk substrates. We are grateful to Bianka Daubertshäuser for technical support with the cultivation of the fungi and to Lukas Kohl for encouraging us to perform this study. We acknowledge financial support from the German Research Foundation (Deutsche Forschungsgemeinschaft).

Financial support. This research has been supported by the German Research Foundation (DFG grant nos. KE 884/8-2, KE 884/16-2 and LE3381/1-1).

Review statement. This paper was edited by Tina Treude and reviewed by Lukas Kohl and one anonymous referee.

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https://doi.org/10.5194/bg-17-3891-2020

Biogeosciences, 17, 3891–3901, 2020


