The stable carbon isotope signature of methane produced by saprotrophic fungi

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Abstract. Methane (CH4) is the most abundant organic compound in the atmosphere with emissions 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 decomposition of plant litter. Although the CH4 production by saprotrophic fungi has been reported, so far, no data for stable carbon isotope values of the emitted CH4 (δ13C-CH4 values) is available. In this study, we measured the δ13C values of CH4 and carbon dioxide (δ13C-CO2 values) emitted by the 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, Festuca rubra) and corn (Zea mays), reflecting 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 mUr to -69 mUr depending on the growth substrate and fungal species. Whilst little apparent carbon isotopic fractionation (in the range of -0.3 mUr to 4.6 mUr) was calculated for 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 mUr 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 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, thermogenic degradation of organic matter and other eukaryotes.
1 Introduction

Methane (CH$_4$) is an important greenhouse gas that is emitted by several abiotic sources (e.g. fossil fuel, biomass burning, geological processes) and biotic sources (e.g. wetlands, agriculture and waste, fresh waters) to the atmosphere (Kirschke et al., 2013; Saunois et al., 2016, 2019). In the past, biotic CH$_4$ 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 CH$_4$ emissions from dead and living plants under oxic conditions (Keppler et al., 2006, 2009) paved the way for the search of new biogenic CH$_4$ sources. Since then, several previously unknown CH$_4$ sources were discovered, including endothelial cells of rat liver (Boros and Keppler, 2019; Ghyczy et al., 2008), plant cell cultures (Wishkerman et al., 2011), marine algae (Klintsch et al., 2019; Lenhart et al., 2016), marine and terrestrial cyanobacteria (Bižić et al., 2020), humans (Keppler et al., 2016) and saprotrophic fungi (Lenhart et al., 2012).

Fungi play a central role in ecosystems by decomposing organic matter and thereby recycling formerly bound carbon and nutrients (Grinhut 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 in these environments (Ralph and Catche, 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 Gloeophyllum trabeum) mainly metabolize cellulose and hemicellulose (Ten Have and Teunissen, 2001; Leonowicz et al., 1999; Valášková and Baldrian, 2006). Fungi have already been determined to be involved in the synthesis of CH$_4$ during wood decay (Beckmann et al., 2011; Mukhin and Voronin, 2007, 2008) by breakdown of large macromolecules to smaller molecules, thereby providing bacteria and archaea with essential substrate.

Elevated levels of CH$_4$ were found in fungus-infected wood stems with oxygen concentrations ranging from 1 to 14 % (Hietala et al., 2015). Here, CH$_4$ production was associated with anoxic microsites in the xylem, indicating that at least part of the CH$_4$ was produced by methanogenic archaea. Nevertheless, Lenhart et al., 2012 demonstrated that basidiomycetes are able to produce CH$_4$ under aerobic conditions without the presence of methanogenic archaea. Therefore, fungi might be an underestimated source of CH$_4$ in the global CH$_4$ cycle.

Stable carbon isotopes (expressed as δ$^{13}$C values) have often been used to investigate sources and sinks of CH$_4$ on the global scale (Whiticar, 1993). As different CH$_4$ sources have characteristic δ$^{13}$C values, δ$^{13}$C-CH$_4$ 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 CH$_4$ in the atmosphere (range 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 δ$^{13}$C-CH$_4$ patterns represent the average of recent inputs by various sources and allows the quantification of respective source strengths (Mikaloff Fletcher et al., 2004b, 2004a).

Additionally, stable isotopes provide information about the formation processes of CH$_4$. Traditionally, three formation categories of δ$^{13}$C-CH$_4$ values have been identified: biogenic, with typical δ$^{13}$C-CH$_4$ values ranging from ~-55 mUr to -70 mUr, thermogenic (ranging from ~-25 mUr to -55 mUr) and pyrogenic (ranging from ~-13 mUr to -25 mUr) (Kirschke et al.,
However, stable isotope values of recently identified CH₄ sources, i.e. human CH₄ emissions (-56 mUr to -95 mUr) (Keppler et al., 2016), plant-derived CH₄ (-52 mUr to -69 mUr) (Keppler et al., 2006), and abiotic UV-induced CH₄ formation by plants (-52 mUr 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 from 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 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*, *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 in 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; Glora, Witten, Germany). The wood chips were dried at 60°C for 48h and stored in a flask (Weck, Hanau, Germany). Grass (*C₃* plant) and corn (*C₄* plant) were selected because of their different stable isotope values. As the metabolic pathway for carbon fixation is biochemically different in *C₃* and *C₄* plants, plant biomass differs in 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 and filled into 2.7 l flasks (Weck, Hanau, Germany) and inoculated with pure fungal submerged cultures under sterile conditions according to Lenhart et al., 2012. After addition of the fungi, the flasks were closed with lids and a rubber band sealing. To allow gas exchange during the growth time of the fungi (about two weeks), a hole in the centre of every lid was fitted with a cotton stopper. Before the start of the incubation experiments, the flasks were aerated under sterile conditions in order to start the incubation at atmospheric CH₄ mixing ratios. Additionally, to seal the flasks airtight the cotton stoppers were replaced by sterile silicone stoppers (Saint-Gobain Performance Plastics, Charny, France).

For the incubation experiments, *P. sapidus* und *L. sulphureus* were incubated on the three substrates, while substrates were incubated as control treatments. Before the incubation experiments, the substrates were sterilized by autoclaving at 121 °C and...
2 bar pressure for 20 minutes. The incubation experiments were conducted as three replicates per treatment. The duration of the incubation accounted for up to 40 h. All incubations were conducted at room temperature (23 ± 1.5 °C). At every sampling point, 40 ml air was taken from the flasks for gas concentration measurements and an additional 40 ml were taken for δ¹³C-CH₄ 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 Exetainers (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 CH₄ were corrected according to the dilution.

When calculating the fungal CH₄ and CO₂ production rates, we subtracted substrate derived CH₄ and CO₂ production rates (determined in the control treatments) from the respective fungi containing samples. Additionally, for the calculation of the fungal production rates only sample points showing a linear increase in CH₄ and CO₂ were taken into account.

To account for differences in the metabolic activity of the fungi, we additionally measured respiration rates, assuming that metabolic activity correlates with respiration and therefore CO₂ emissions of the fungi. Hence, we related fungal derived CH₄ emissions to respiration by calculating the CH₄ : CO₂ emission ratio.

2.3 Analysis of CH₄ and CO₂ 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 CH₄ and CO₂, respectively. The detector temperatures were set at 300 °C (FID) and 350 °C (ECD). Five reference gases (Deuste Steininger GmbH) were used for calibrating the GC-system. The reference gases were in the range of 1 parts per million by volume (ppmv) to 21 ppmv and 304 ppmv to 40,000 ppmv for CH₄ and CO₂, 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 compared to the standard substance Vienna PeeDee Belemnite (V-PDB) (Eq. (1)).

\[
\delta^{13}C = \frac{^{13}C}{^{12}C}_{\text{sample}} - 1
\]

The apparent fractionation (ε_app) between fungal δ¹³C-CH₄ or δ¹³C-CO₂ values and the δ¹³C values of the substrates was calculated according to Eq. (2).

\[
\epsilon_{\text{app CH₄ or CO₂}} = \frac{(\delta^{13}C + 1)_{\text{fungal CH₄ or CO₂}}}{(\delta^{13}C + 1)_{\text{substrate}}} - 1
\]
We follow the proposal of Brand and Coplen, 2012 and use the term ‘urey’ (Ur) 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 preconcentration unit for CH\(_4\) measurements and an autosampler A200S (CTC Analytics, Zwingen, Switzerland) for CO\(_2\) analysis. The GC was equipped with a CP-PoraPLOT Q capillary column (Varian, Palo Alto, USA) (27.5 m x 0.25 mm i.d., film thickness 8 µm). The GC was operated with an injector temperature of 200°C, isothermal 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 Delta\(^{\text{PLUS}}\)XL isotope ratio mass spectrometer (ThermoQuest Finnigan, Bremen, Germany) via an oxidation reactor and a GC Combustion III Interface (ThermoQuest Finnigan, Bremen, Germany).

The oxidation reactor was employed with the following properties: ceramic tube (Al\(_2\)O\(_3\)), length 320 mm, 1.0 mm i.d., with Ni/Pt wires inside activated by oxygen, reactor temperature 960 °C. For CH\(_4\) measurements with the preconcentration 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 introduced into the IRMS system via an open split. The monitor 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 MPI for Biogeochemistry in Jena, Germany). All δ\(^{13}\)C values were corrected using two CH\(_4\) reference standards (Isometric instruments, Victoria, Canada) with δ\(^{13}\)C values of -23.9 ± 0.2 mUr and -54.5 ± 0.2 mUr that were calibrated against IAEA and NIST reference substances. The normalization of the sample values was done according to Paul et al., 2007.

2.6 Bulk isotope analysis of fungal substrates

Stable carbon isotope values of the bulk substrate were measured using an Elemental Analyzer Flash EA 11112 (Thermo Fischer Scientific, Germany) coupled to a Delta V IRMS (Thermo Fischer Scientific, Germany). Therefore, 0.06 mg of the substrate was put into a tin cup and combusted in the Elemental Analyzer. The resulting gases were separated in a GC by a CP-PoraPLOT Q capillary column (Varian, Palo Alto, USA) (27.5 m x 0.25 mm i.d., film thickness 8 µm) and then reached the Delta V IRMS via a Conflo IV Universal Continuous Flow Interface (Thermo Fischer Scientific, Germany). Isotope values were corrected using USGS 40 and USGS 41 standards.

2.7 Determination of isotopic source signature of CH\(_4\) and CO\(_2\) applying Keeling plots

For the determination of δ\(^{13}\)C source values of CH\(_4\) and CO\(_2\) the Keeling plot method was used (Keeling, 1958; Pataki et al., 2003) (Eq. (3)): 
\[ \delta^{13}C_a = c_b (\delta^{13}C_b - \delta^{13}C_s) \left( \frac{1}{c_a} \right) + \delta^{13}C_s \]  

(3)

where \( c_a \) is the mixing ratio of CH\(_4\)/CO\(_2\) in the headspace, \( \delta^{13}C_a \) is the \( \delta^{13}C \) value of CH\(_4\)/CO\(_2\) in the headspace, \( c_b \) is the mixing ratio of background CH\(_4\)/CO\(_2\), \( \delta^{13}C_b \) is the \( \delta^{13}C \) value of background CH\(_4\)/CO\(_2\) and \( \delta^{13}C_s \) the \( \delta^{13}C \) source value of the CH\(_4\)/CO\(_2\). For a more detailed description of the application of Keeling plots for determination of CH\(_4\) source signature we refer to the study by Keppler et al., 2016.

\( \delta^{13}C \)-CH\(_4\) source signatures were calculated after the Keeling plot method for each flask. Results of the Keeling plots are then given as the arithmetic mean of the three individual flasks per treatment with standard deviations (n=3).

\( \delta^{13}C \)-CH\(_4\) source signatures of each flask of \( P. \) sapidus and \( L. \) sulphureus grown on pine were corrected for CH\(_4\) emissions and \( \delta^{13}C \)-CH\(_4\) values of the “pine” control samples using the following mass balance approach (Eq. (4)).

\[ \delta^{13}C_{fungi \ corrected} = \frac{(P(CH_4)_{fungi} \times \delta^{13}C_{fungi}) - (P(CH_4)_{pine} \times \delta^{13}C_{pine})}{(P(CH_4)_{fungi} - P(CH_4)_{pine})} \]  

(4)

, where \( P(CH_4)_{fungi/pine \ wood} \) is the CH\(_4\) emitted by the fungi or pine wood and \( \delta^{13}C_{fungi/pine \ wood} \) is the \( \delta^{13}C \)-CH\(_4\) source signature of the fungi or pine wood derived from Keeling 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 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 of \( \delta^{13}C \)-CH\(_4\) values due to the small increase of the CH\(_4\) mixing ratio compared to the background CH\(_4\) mixing ratio. Therefore, the low R\(^2\) does not necessarily indicate a weaker relationship between CH\(_4\) mixing ratio and \( \delta^{13}C \)-CH\(_4\).

2.8 Statistics

Mixing ratios and production rates of CH\(_4\), CO\(_2\), \( \delta^{13}C \)-CH\(_4\) and \( \delta^{13}C \)-CO\(_2\) values and \( \delta^{13}C \) source values are presented as arithmetic mean of three independent replicates with standard deviations (SD; n = 3). Linear regression analysis, arithmetic means and SDs were calculated using Excel (Microsoft Excel for Office 365 MSO). Two-way analysis of variance (ANOVA) (SigmaPlot 12.2.0.45, USA) were carried out to test for “species” and “substrate” related effects on \( \delta^{13}C \)-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 δ^{13}C source values of fungal derived CH₄ with values known for other CH₄ sources from the literature.

3.1 Release of CH₄ and CO₂ from P. sapidus and L. sulphureus

![Figure 1](image)

**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 standard deviation SD (n=3).

All incubation experiments in which fungi were grown on different substrates showed a significant increase in CH₄ compared to the respective substrate control (Fig. 1 a, c). Calculated emission rates for CH₄ and CO₂ are presented in Table 1. *L. sulphureus* grown on grass (7.5 ± 1.3 nmol h⁻¹) showed the highest emission rate of CH₄, 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 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 CH$_4$ and CO$_2$ ($\mu$mol CH$_4$ : mol CO$_2$). CO$_2$ production thereby reflects the amount of fungal biomass and is also an indicator for the metabolic activity of the fungi.

The control flasks did not show significant changes in their CH$_4$ and CO$_2$ mixing ratios over time, except for CH$_4$ in pine wood controls (1.3 ± 0.1 nmol h$^{-1}$). However, in the control flasks of corn small CH$_4$ emission rates of 0.25 ± 0.01 nmol h$^{-1}$ were observed and in the control ‘grass’ the CH$_4$ mixing ratio slightly decreased over time (-0.05 ± 0.04 nmol h$^{-1}$). Whilst the pine wood and corn control flasks showed a small increase in the CH$_4$ mixing ratio, they did not show an increase in CO$_2$ mixing ratios. These data rule out a contamination by microbial heterotrophs, as this would cause a measurable CO$_2$ increase within the flasks. The CH$_4$ increase in the substrate controls might be attributed to CH$_4$ release by dead plant material as it 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, Lenhart et al., 2012 clearly showed that with the performed method of cultivation of fungi and incubation experiments no methanogenic archaea were present, using three different methods (Fluorescence in situ hybridization (FISH), confocal laser scanning microscopy (CLSM) and quantitative real time PCR). Furthermore, CH$_4$ and CO$_2$ release and the CH$_4$ : CO$_2$ 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 in our investigations no contamination with bacteria or methanogenic archaea was present.

For _P. sapidus_ grown on corn and _L. sulphureus_ grown on grass, no further linear increase in CH$_4$ was observed after 22 h and 10 h, respectively. This might be due to a reduced decay of organic matter and slower fungal metabolism because of higher CO$_2$ and lower O$_2$ mixing ratios.

A drastic increase in CO$_2$ mixing ratios relative to the controls was observed in all flasks containing fungi (Fig. 1 b, d). The CO$_2$ emission rates are shown in Table 1. CO$_2$ production rates ranged from 176 ± 4 µmol h$^{-1}$ to 2910 ± 410 µmol h$^{-1}$ for _P. sapidus_ grown on grass and _P. sapidus_ grown on corn, respectively. These highly variable CO$_2$ production rates might reflect different fungal biomass and metabolic activity (mineralisation of organic matter). In the control treatments, tiny increases in the CO$_2$ mixing ratio were detected ranging from 0.64 ± 0.12 µmol h$^{-1}$ to 0.91 ± 0.14 µmol h$^{-1}$. Only one flask (corn control) showed a somewhat higher increase in CO$_2$ (7.76 µmol h$^{-1}$), which is most likely caused by microbial contamination of the flask. However, no increase in the CH$_4$ mixing ratio was detected (see supplementary material). Therefore, this control flask was excluded from further calculations.

Mean CH$_4$ and CO$_2$ emission rates and CH$_4$ : CO$_2$ emission ratios of all treatments are presented in Table 1. Higher ratios indicate a higher CH$_4$ production during decay of the substrates. Thereby, both fungal species and substrate affect the CH$_4$ : CO$_2$ emission ratio ($p<$0.001). For _P. sapidus_, CH$_4$ : CO$_2$ emission ratios are more variable (1.4 to 8.0 µmol CH$_4$/mol CO$_2$) compared to _L. sulphureus_ (6.7 – 9.6 µmol CH$_4$/mol CO$_2$). 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 CH$_4$ are still unknown, although compounds such as the sulphur-bound methyl-group of methionine and glucose have been identified to act as carbon precursors of fungal-derived CH$_4$ (Lenhart et al., 2012).
Lenhart et al., 2012 found CH$_4$ : CO$_2$ ratios of fungi that ranged between 8 μmol CH$_4$/mol CO$_2$ and 17 μmol CH$_4$/mol CO$_2$, which is in the same order of magnitude as the CH$_4$ : CO$_2$ ratios determined in this study. It should be noted that CH$_4$ : CO$_2$ ratios of Lenhart et al., 2012 were given in ppbv CH$_4$ : % CO$_2$ and for better comparability CH$_4$ : CO$_2$ ratios were converted to fit the units used in this study (μmol CH$_4$ : mol CO$_2$).

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**Table 1:** CH$_4$ and CO$_2$ production rates and molar CH$_4$ : CO$_2$ emission ratios of the fungi incubated on different substrates. Values are presented as mean values of three independent replicates with SD (n = 3), except for the control “corn” (n=2).

| Fungi       | Substrate | CH$_4$ production rate [nmol h$^{-1}$] | CO$_2$ production rate [μmol h$^{-1}$] | CH$_4$ : CO$_2$ ratio [μmol/mol] |
|-------------|-----------|---------------------------------------|---------------------------------------|----------------------------------|
| *P. sapidus*| pine      | 2.5 ± 0.2                             | 901 ± 79                              | 2.8 ± 0.4                        |
|             | grass     | 1.4 ± 0.5                             | 176 ± 4                               | 8.0 ± 2.8                        |
|             | corn      | 4.4 ± 1.9                             | 2910 ± 419                            | 1.4 ± 0.5                        |
| *L. sulphureus* | pine  | 6.2 ± 0.3                             | 724 ± 42                              | 8.6 ± 1.0                        |
|             | grass     | 7.5 ± 1.3                             | 771 ± 103                             | 9.6 ± 0.5                        |
|             | corn      | 2.6 ± 0.1                             | 385 ± 20                              | 6.7 ± 0.4                        |
| control     | pine      | 1.3 ± 0.1                             | 0.64 ± 0.12                           | -                                |
|             | grass     | -0.05 ± 0.04                          | 0.91 ± 0.14                           | -                                |
|             | corn      | 0.25                                  | 0.66                                  | -                                |
3.2 Stable carbon isotope values of CH$_4$ and CO$_2$

**Figure 2**: Stable carbon isotope values of CH$_4$ and CO$_2$ of *P. sapidus* (a, c) and *L. sulphureus* (b, d) grown on pine, grass, and corn. Values are presented as mean values with SD (n=3), except for $\delta^{13}$CO$_2$ values of *L. sulphureus* grown on corn (n=2).

Stable carbon isotope values of CH$_4$ and CO$_2$ measured from the incubation experiments are presented in Fig. 2. All incubations show a trend towards more negative $\delta^{13}$C-CH$_4$ values (less $^{13}$C) with time except for *P. sapidus* grown on corn, where a tendency towards more positive $\delta^{13}$C-CH$_4$ values was observed (Fig. 2 a, b). During the incubation, $\delta^{13}$C-CH$_4$ values changed from -47.7 ± 0.1 mUr (for incubation of *P. sapidus* grown on pine/grass) and -48.2 ± 0.1 mUr (for incubation of *P. sapidus* grown on corn and *L. sulphureus* grown on pine/grass/corn) to -53.0 ± 0.7 mUr (*P. sapidus* grown on pine), -48.7 ± 0.3 mUr (*P. sapidus* grown on grass), -45.8 ± 1.2 mUr (*P. sapidus* grown on corn), -55.1 ± 0.4 mUr (*L. sulphureus* grown on pine), -55.4 ± 0.4 mUr (*L. sulphureus* grown on grass) and -49.9 ± 0.4 mUr (*L. sulphureus* grown on corn). The controls showed no significant shift in $\delta^{13}$C-CH$_4$ values except for the control “pine”, where an increase in the CH$_4$ mixing ratio along with more
negative values of $\delta^{13}$C-CH$_4$ values occurred over time. This was accounted for when calculating the $\delta^{13}$C-CH$_4$ source signatures for $P$. sapidus grown on pine and $L$. sulphureus grown on pine (see materials and methods 2.7).

The $\delta^{13}$C-CO$_2$ values showed a trend towards more negative values within the first three to four hours of incubation (Fig. 2 c, d). After this time only minor changes of the $\delta^{13}$C-CO$_2$ values occurred. Final $\delta^{13}$C-CO$_2$ values of the incubation were -24.9 ± 0.6 mUr ($P$. sapidus grown on pine), -28.6 ± 0.9 mUr ($P$. sapidus grown on grass), -12.0 ± 0.3 mUr ($P$. sapidus grown on corn), -24.1 ± 0.1 mUr ($L$. sulphureus grown on pine), -27.7 ± 0.5 mUr ($L$. sulphureus grown on grass) and -13.0 ± 0.5 mUr ($L$. sulphureus grown on corn).

**Table 2:** Calculated $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-CO$_2$ source signatures, $\delta^{13}$C values of the substrates, and $\varepsilon_{\text{app, CH}_4}$ and $\varepsilon_{\text{app, CO}_2}$. Values are presented as mean values with the SD (n=3).

| Fungi  | Substrate | $\delta^{13}$C-CH$_4$ source [mUr] | $\delta^{13}$C-CO$_2$ source [mUr] | $\delta^{13}$C substrate [mUr] | $\varepsilon_{\text{app, CH}_4}$ [mUr] | $\varepsilon_{\text{app, CO}_2}$ [mUr] |
|--------|-----------|----------------------------------|----------------------------------|-------------------------------|---------------------------------|---------------------------------|
| $P$. sapidus | pine | -65.3 ± 1.1 | -24.1 ± 0.1 | -38.4 ± 1.2 | 4.0 ± 0.1 |
|        | grass  | -52.9 ± 1.6 | -27.4 ± 1.3 | -21.8 ± 1.7 | 4.6 ± 1.3 |
|        | corn   | -39.8 ± 2.0 | -12.0 ± 0.3 | -28.5 ± 2.0 | -0.3 ± 0.3 |
| $L$. sulphureus | pine | -61.4 ± 0.5 | -25.0 ± 0.5 | -34.4 ± 0.6 | 3.0 ± 0.4 |
|        | grass  | -69.2 ± 1.9 | -29.0 ± 0.5 | -38.6 ± 2.0 | 2.9 ± 0.5 |
|        | corn   | -53.4 ± 1.1 | -12.8 ± 0.3 | -42.2 ± 1.1 | -1.1 ± 0.3 |
| control | pine |                    | -28.0 ± 0.5 |                            |                                |
|         | grass  |                    | -31.5 ± 0.6 |                            |                                |
|         | corn   |                    | -11.7 ± 0.1 |                            |                                |
3.3 δ^{13}C-CH₄ and δ^{13}C-CO₂ source signatures of fungi

![Keeling plots](image)

**Figure 3:** Keeling plots shown for *P. sapidus* (a) and *L. sulphureus* (b) grown on three substrates. Sample points in the graphs are given as the arithmetic mean of δ^{13}C-CH₄ or δ^{13}C-CO₂ values with SD (n=3) on the y-axis and the arithmetic mean of the inverted mixing ratio of CH₄ or CO₂ with SD (n=3) on the x-axis.

The δ^{13}C-CH₄ source signatures determined via a Keeling plot analysis (Fig. 3) that range from -69.2 ± 1.9 mUr (*L. sulphureus* grown on grass) to -39.8 ± 2.0 mUr (*P. sapidus* grown on corn) are presented in Table 2. Average δ^{13}C-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 decomposing different components of the growth substrates, as *P. sapidus* belongs to white rot fungi and *L. sulphureus* is a brown rot fungus. However, detailed investigations of the metabolic pathways leading to CH₄ formation were beyond the scope of this study.

Furthermore, a significant effect of the growth substrate on δ^{13}C-CH₄ source signatures was observed (*p*<0.001). δ^{13}C-CH₄ source signatures by *P. sapidus* were more positive compared to those of *L. sulphureus* when grown on grass (Δ=16.3 mUr) and corn (Δ=13.6 mUr) (Fig. 4). When grown on pine wood, δ^{13}C-CH₄ source signatures were similar with *P. sapidus* showing slightly more negative values (Δ=-3.9 mUr). Methane emitted by both fungi grown on corn was generally more enriched in ^{13}C (less negative δ^{13}C-CH₄ source values) compared to the fungi grown on pine wood and grass. This might be easily explained by the δ^{13}C values of the growth substrates corn (-11.7 mUr, typical for C₄-plants) being roughly 20 mUr less negative in their δ^{13}C values compared to the C₃-plants pine wood (-28.0 mUr) and grass (-31.5 mUr).
Comparison of calculated $\delta^{13}$C-$\text{CH}_4$ 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 to the respective substrates (Fig. 4a). Based on this data we further calculated the apparent fractionation ($\varepsilon_{\text{app CH}_4}$) between the $\delta^{13}$C-$\text{CH}_4$ source signatures and the bulk $\delta^{13}$C values of the growth substrates. The apparent fractionation was calculated as up to the present no metabolic pathway for the formation of CH$_4$ in fungi is known and therefore currently only the initial $\delta^{13}$C signatures of the substrates and the calculated $\delta^{13}$C-$\text{CH}_4$ source signatures of the fungi can be compared. The values of $\varepsilon_{\text{app CH}_4}$ that range from -21.8 mUr ($P. \text{sapidus}$ grown on grass) to -42.2 mUr ($L. \text{sulphureus}$ grown on corn) are presented in Table 2. When grown on pine wood $\varepsilon_{\text{app CH}_4}$ values are similar for $P. \text{sapidus}$ (-38.4 ± 1.2 mUr) and $L. \text{sulphureus}$ (-34.4 ± 0.6 mUr). The differences in $\varepsilon_{\text{app CH}_4}$ values between both fungal species are distinct when grown on grass ($P. \text{sapidus}$: -21.8 ± 1.7 mUr, $L. \text{sulphureus}$: -38.6 ± 2.0 mUr) and corn ($P. \text{sapidus}$: -28.5 ± 2.0 mUr, $L. \text{sulphureus}$: -42.2 ± 1.1 mUr).

The calculated $\delta^{13}$C-$\text{CO}_2$ source signatures of both fungi (Table 2) range from -29.0 ± 0.5 mUr ($L. \text{sulphureus}$ grown on grass) to -12.0 ± 0.3 mUr ($P. \text{sapidus}$ grown on corn). $\delta^{13}$C-$\text{CO}_2$ source signatures are in a similar range for both fungi for all three substrates. However, CO$_2$ emitted by $L. \text{sulphureus}$ is slightly more depleted in $^{13}$C for all three substrates compared to $P. \text{sapidus}$. Hence, an effect of fungal species on the stable carbon isotope values of CO$_2$ is significant ($p=0.008$). Also, the used substrates were found to influence $\delta^{13}$C-$\text{CO}_2$ values significantly ($p<0.001$).

The $\delta^{13}$C-$\text{CO}_2$ 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-$\text{CO}_2$ values are slightly less negative (a few mUr) compared to the bulk substrate. This observation is rather unexpected, as $\delta^{13}$C-$\text{CO}_2$ values are usually more negative with respect to $\delta^{13}$C values of growth substrates due to fractionation during the metabolism (Bowling et al., 2008). However, when
grown on corn δ¹³C-CO₂ source signatures by both fungi are more negative compared to the substrate and calculated ε_app CO₂ values (Table 2) are -1.1 ± 0.3 mUr and +4.6 ± 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 δ¹³C-CH₄ and δ¹³C-CO₂ values released by both fungi. While the δ¹³C-CO₂ source signatures are similar to the δ¹³C values of the substrate (with ε_app CO₂ values up to 4.6 mUr), the δ¹³C-CH₄ source signatures deviate strongly from the respective substrate, with ε_app CH₄ values of up to -42.2 mUr. This either indicates that metabolic pathways leading to the formation of CH₄ and CO₂ have different fractionation and/or that fungal CH₄ and CO₂ are derived from different precursor compounds of the respective substrate. The growth substrates used for this study (pine wood, grass, corn) contain distinct amounts of cellulose, hemicellulose, lignin and other compounds at different proportions (in contrast to only using pure glucose or cellulose as growth substrate). Hence, the δ¹³C-CH₄ and δ¹³C-CO₂ 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 used growth substrates provide a first solid basis for the potential range of δ¹³C-CH₄ values that might occur in nature.

### 3.4 Fungal δ¹³C-CH₄ values compared with known CH₄ sources

Figure 5 compares the δ¹³C-CH₄ values emitted by fungi in relation to other known CH₄ sources in the environment that have been reported from the literature. The red bars indicate typical biogenic (formerly only considered to be produced by archaea) CH₄ sources with emissions from wetlands, ruminants, landfills and rice paddies where δ¹³C-CH₄ values are usually ranging from -85 mUr to -40 mUr. Abiotic CH₄ sources (including thermogenic or pyrolytic processes) stemming from natural gas, coal mining and biomass burning are characterized by less negative δ¹³C values usually ranging from -55 mUr to -20 mUr. In addition gas hydrates which might be formed by both microbial and abiotic processes cover a wide range of δ¹³C values (-29 mUr to -73 mUr), depending on its forming mechanisms (Kvenvolden, 1995). The δ¹³C source signatures of plant derived CH₄ have been reported to be in the range of -72 mUr to -45 mUr (Keppler et al., 2006; Vigano et al., 2009) depending on the photosynthetic pathways (C₃, C₄ or CAM). Furthermore, there is a tendency towards more negative δ¹³C-CH₄ values when the respective plant was treated with UV radiation (Vigano et al., 2009). δ¹³C-CH₄ source signatures of humans which might include both formation by microbes in the gut but also from cellular processes show a rather wide range with values between -95 mUr 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 δ¹³C-CH₄ source values from -69 mUr to -40 mUr. This range overlaps with other eukaryotic sources, most microbial CH₄ sources and even some abiotic CH₄ sources such as natural gas or emissions from coal mining.
Figure 5: Range of $\delta^{13}$C-$\text{CH}_4$ 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 taken from (Brownlow et al., 2017; Keppler et al., 2006, 2016; Kvenvolden, 1995; Nisbet et al., 2016; Quay et al., 1999; Vigano et al., 2009).

4 Conclusion

This study provided the first analysis of stable carbon isotope values of CH$_4$ emitted by two saprotrophic fungi that were grown on three different substrates. $\delta^{13}$C-CH$_4$ and $\delta^{13}$C-CO$_2$ source values were found to be dependent on the fungal species, as well as the substrates decomposed by the fungi. $\delta^{13}$C-CH$_4$ source values of the fungi were found to be in the range of -69 mUr to -40 mUr and therefore overlap with $\delta^{13}$C-CH$_4$ values reported for other CH$_4$ sources such as methanogenic archaea, eukaryotes and from abiotic CH$_4$ sources (e.g. natural gas, coal mining). Stable carbon isotope values of CH$_4$ in combination with flux measurements are often applied for a better understanding of regional and global CH$_4$ cycling. However, in recent years it has become clear that many biogenic CH$_4$ sources include complex 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 CH$_4$ source has to be expected (e.g. methanogenic archaea, fungi, cyanobacteria or plants) gets increasingly complicated as distinguishing between each individual source solely by 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 CH$_4$ ($\delta^2$H-CH$_4$ values) or even applications of clumped isotopes might prove suitable tools for better distinction between different CH$_4$ sources and thus to better constrain the global CH$_4$ budget.

Data availability. We provide the data in heiDATA, which is an institutional repository for research data of Heidelberg University (https://doi.org/10.11588/data/DQYPMC).

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.

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