Supplementary information

Fungal Methane Production Controlled by Oxygen Levels and Temperature

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Text S1. Oxygen and temperature dependency of CO_2 mixing ratios during fungal incubations

During all fungal incubations, CO_2 mixing ratios within the flasks significantly increased to up to 35% (*L. sulphureus* grown on pine wood at 27°C), indicating the metabolic activity of the investigated fungi (Figure S1). Due to logistic constraints, CO_2 mixing ratios could not be sampled at high resolution as for CH_4 or O_2 mixing ratios. Nonetheless, it was evident that CO_2 mixing ratios increased at a considerably higher rate when O_2 was present in the flasks, compared to conditions where O_2 had been previously consumed by the fungi (e.g., for *P. sapidus* grown on pine wood that showed CO_2 emission rates of 0.39 mmol h^{-1} when O_2 was present, while rates accounted for 0.01 mmol h^{-1} when O_2 was consumed prior; Figures S1C, F, I). However, CO_2 levels continued to rise even after O_2 depletion, however at a slower rate, suggesting reduced metabolic activity by the fungi (e.g., Figure S1C). This observation aligns with findings from Mukhin and Diyarova [31] which demonstrated that basidiomycetes also emit CO_2 in environments with low to no measurable O_2.

Temperature also had a noticeable effect on fungal CO_2 emissions. Incubations at 17°C always resulted in smaller CO_2 increases compared to those at 27 or 40°C, regardless of the fungal species or growth medium (e.g., for *P. sapidus* grown on pine wood, where CO_2 emission rates accounted for 0.39 mmol h^{-1} and 0.16 mmol h^{-1} at temperatures of 27 °C and 17°C, respectively). The difference in the rate of increase in fungal CO_2 emissions was particularly pronounced for *L. sulphureus* grown on pine wood, as opposed to *P. sapidus* grown on the same medium. This suggests that the metabolic activity of *L. sulphureus* was more temperature-dependent than that of *P. sapidus*.

In contrast to fungal incubations, CO_2 mixing ratios of control incubations (explained in more detail in Text S2) showed relatively small increases across different temperatures, indicating that CO_2 emission were closely associated with fungal metabolic activity, similarly as reported by [17,18].

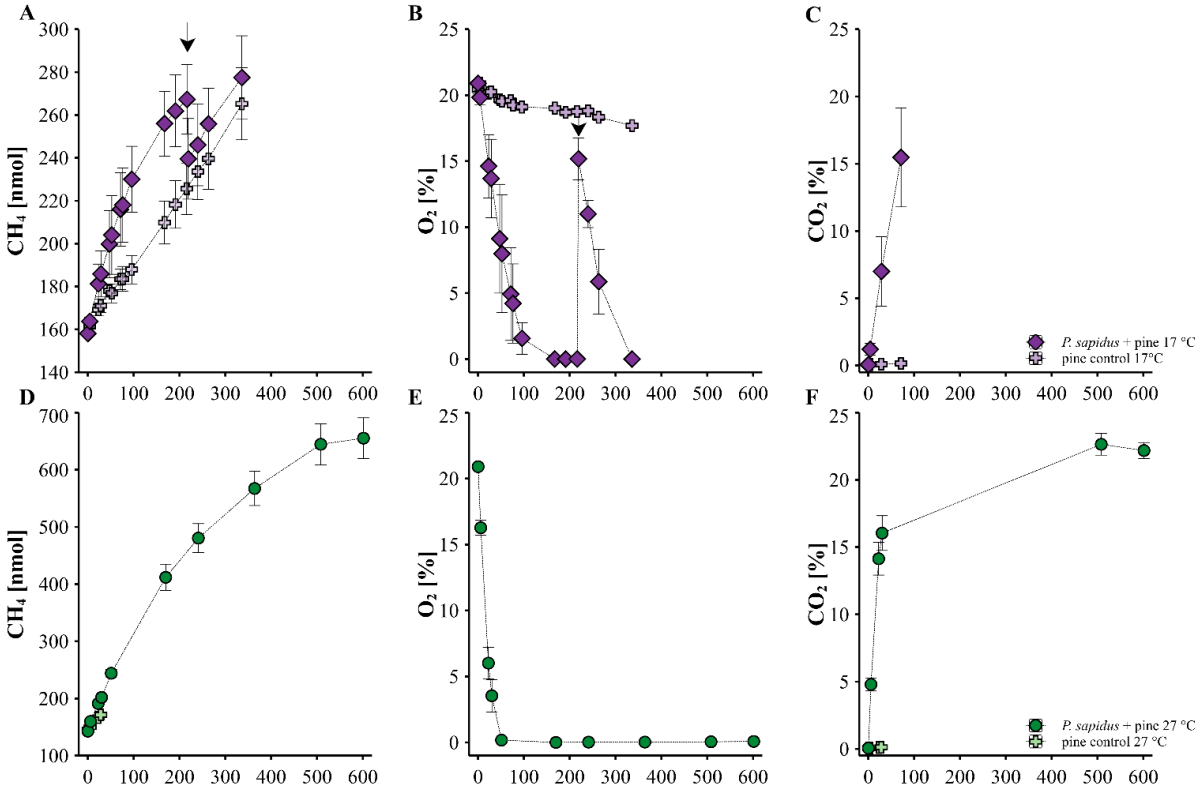

Figure S1: Changes of CH_4 amounts (A, D) as well as O_2 (B, E) and CO_2 levels (C, F), respectively, in the flasks during incubation of *P. sapidus* grown on pine wood at 17 and 27 °C. Black arrows indicate the points of O_2 addition to the individual flasks containing fungi. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4).

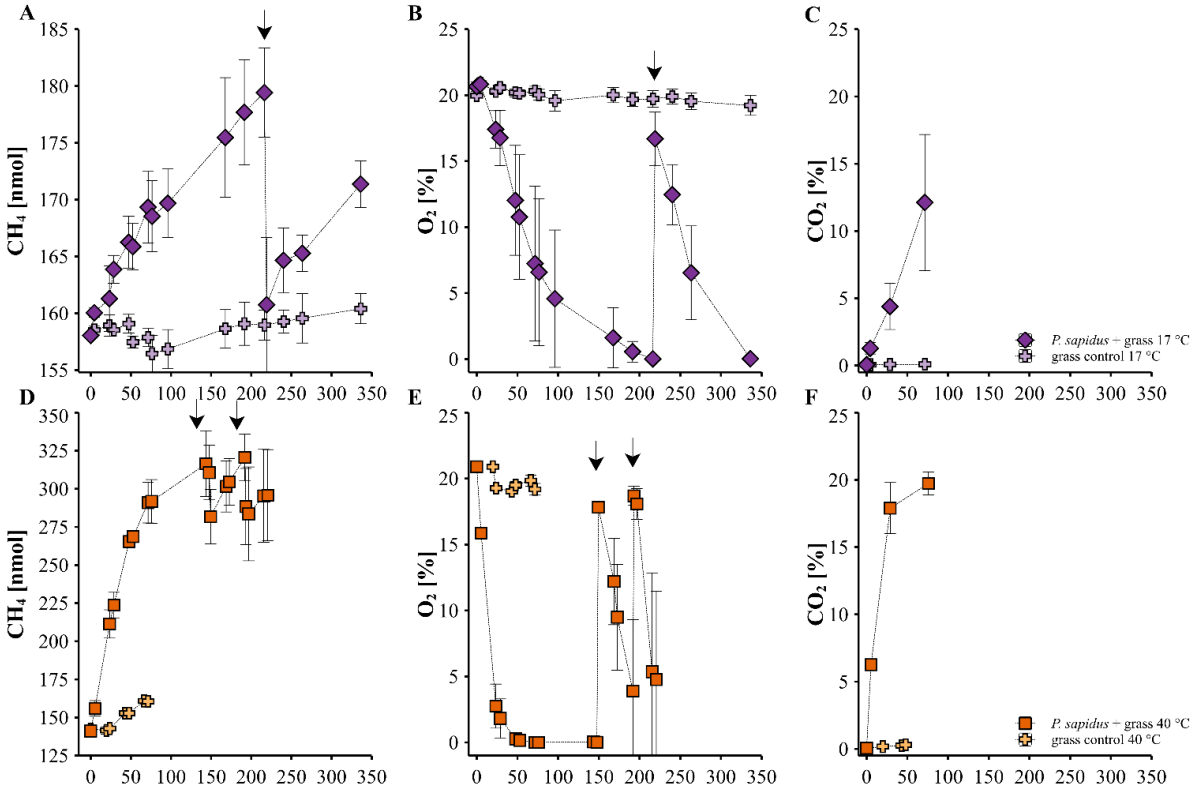

Figure S2: Changes of CH_4 amounts (A, D) as well as O_2 (B, E) and CO_2 levels (C, F), respectively, in the flasks during incubation of *P. sapidus* grown on grass at 17 and 40 °C. Black arrows indicate the points of O_2 addition to the individual flasks containing fungi. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4).

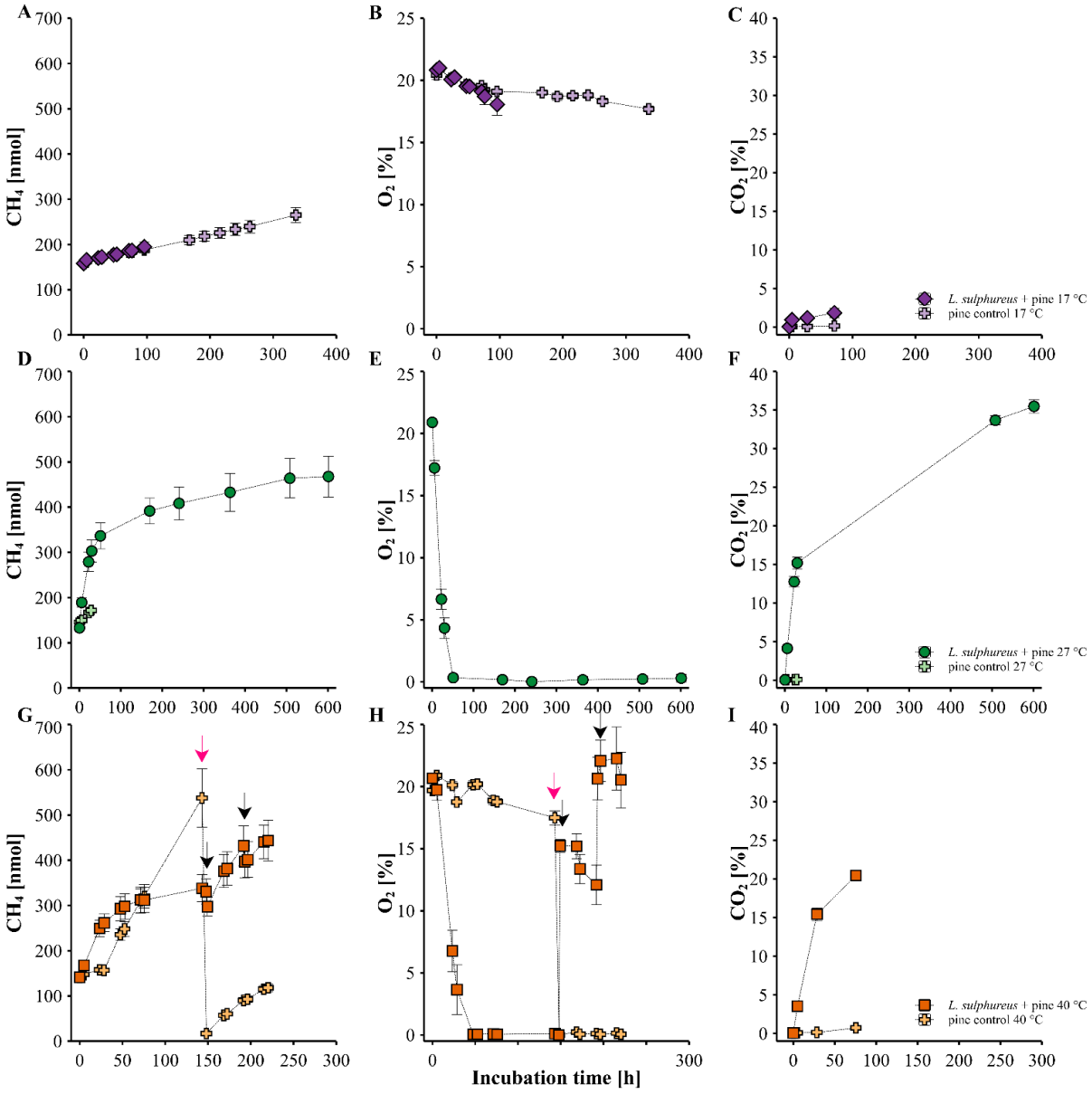

Figure S3: Changes of CH_4 amounts (A, D, G) as well as O_2 (B, E, H) and CO_2 levels (C, F, I), respectively, in the flasks during incubation of *L. sulphureus* grown on pine wood at 17, 27, and 40 °C. Black arrows indicate the points of O_2 addition to the individual flasks containing fungi, while pink arrows indicate O_2 removal by flushing of the incubation flask with helium. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4).

Text S2. Changes in CH_4, O_2 and CO_2 levels during incubation of pine wood and grass controls

Figure S2 displays the changes in CH_4 levels, as well as O_2 and CO_2 concentrations, during control incubations of pine wood at 17, 27, and 40°C (A, B, C) and grass at 17°C and 27°C (D, E, F). Both pine wood and grass controls exhibited an increase in CH_4 levels, which increased with temperature. For pine wood controls, the highest CH_4 increase rate was observed at 40°C (3.26 ± 0.53 nmol h^{-1}), surpassing the rates at 27°C (0.91 ± 0.09 nmol h^{-1}) and 17°C (0.30 ± 0.05 nmol h^{-1}). For grass controls a similar trend was observed with higher CH_4 increase rates at 27°C (0.31 ± 0.01 nmol h^{-1}) compared with 17°C (0.01 nmol h^{-1}). Although CH_4 levels rose within the flasks, it is noteworthy that increases were 0.5 to 14 times higher in the presence of either of the two fungal species grown on the respective substrates and temperature. Measurements for pine wood controls at 40°C were conducted with and without O_2 (Fig. S2A), with the O_2-free treatment's headspace being replaced by helium. Notably, CH_4 formation rates in the O_2-free control were 60% lower than those in the presence of O_2. The reason for this observation is currently still unclear and should be addressed in future research.

A similar trend was noted for O_2 and CO_2 mixing ratios during the incubation of controls. Oxygen concentrations slightly declined for the pine wood controls, more so at 40°C (0.02 mmol h^{-1}) compared with 17°C (0.01 mmol h^{-1}), while CO_2 concentrations increased at a higher rate at 40°C (0.006 mmol h^{-1}) than at 27°C (0.002 mmol h^{-1}) and 17°C (0.001 mmol h^{-1}). During the incubation of grass controls only negligible changes in O_2 mixing ratios were observed, while CO_2 increased at a higher rate at 27°C (0.003 mmol h^{-1}) compared to 17°C (0.0003 mmol h^{-1}). Rates were up to 18 times lower when compared to incubation with fungi for O_2 consumption rates and between 8 to 390 times lower when compared to CO_2 emissions for the respective substrate and fungi. Like in the fungi incubation experiments, the substrates were sterilized before incubation. Thus, the temperature dependent changes in CH_4 levels, along with the much smaller changes in O_2 and CO_2 mixing ratios point to an abiotic formation of CH_4 and CO_2.

These observations align with findings by Lenhart *et al*. and Schroll *et al.* [17,18], which also reported a small CH_4 production in control media (pine-, spruce-, birch-, beech- and oak wood as well as grass and corn), hinting at a potential abiotic source for this compound. Previous research has linked abiotic CH_4 formation to factors such as UV-B radiation (e.g., [48–50]), temperature [50,51], the presence of H_2O_2 [52], and iron-oxo catalysis (e.g., [53–55]) through the interaction of ROS and iron (II) in the presence of methylated sulfur and nitrogen compounds. Furthermore, Hädeler *et al.* [56] demonstrated that, besides CH_4 and CO_2, other C_1 and C_2 compounds such as methanol, formate or ethane can be generated from methyl groups in organic matter through iron oxide-mediated methyl radical formation. Nevertheless, the exact mechanisms of the observed abiotic CH_4 formation in our control experiments is currently unknown. This phenomenon demands future investigation, since both the observed abiotic formation of CH_4 and CO_2 as well as potential formation of other C_1 and C_2 compounds could have a strong potential to contribute to the carbon and nitrogen cycle in various environments.

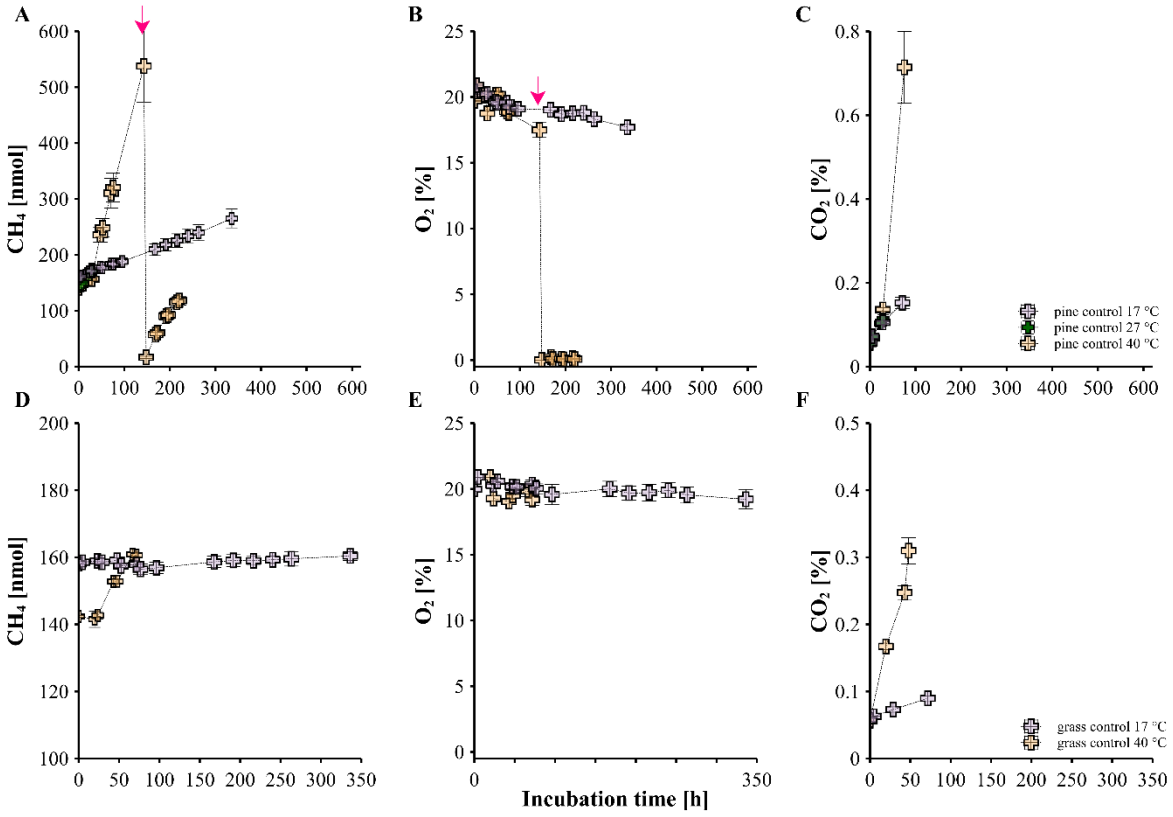

Figure S4: Changes of CH_4 amounts as well as O_2 and CO_2 levels in the flasks during control incubation of A), B), C) pine wood at 17, 27, and 40 °C and D), E), F) grass at 17 and 40 °C. The pink arrow indicates O_2 removal by flushing of the incubation flask with helium. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 3 to 4).

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