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Fungal Methane Production Controlled by Oxygen Levels and Temperature

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Abstract: Saprotrophic fungi, key players in global carbon cycling, have been identified as methane (CH_4) sources not yet accounted for in the global CH_4 budget. This study, for the first time, explores the influence of oxygen (O_2) and temperature on CH_4 production by two fungi, *Laetiporus sulphureus* and *Pleurotus sapidus*. To explore the relationship between these parameters and fungal CH_4 formation, we examined CH_4 formation under varying O_2 levels (0 to 98%) and temperatures (17, 27, and 40 °C) during fungal growth on pine wood, beech wood, and grass under sterile conditions. Our findings show that fungal CH_4 formation strongly depends on O_2 levels. Methane formation was highest when O_2 levels exceeded 5%, whilst no CH_4 formation was observed after complete O_2 consumption. Reintroducing O_2 immediately resumed fungal CH_4 production. Methane formation normalized to O_2 consumption (CH_{4_norm}) showed a different pattern. *L. sulphureus* showed higher CH_{4_norm} rates with higher O_2 levels, whereas *P. sapidus* showed elevated rates between 0 and 5%. Temperature also significantly influenced CH_4 and CH_{4_norm} rates, with the highest production at 27 °C, and comparatively lower rates at 17 and 40 °C. These findings demonstrate the importance of O_2 levels and temperature in fungal CH_4 emissions, which are essential for refining CH_4 source predictions.

Keywords: aerobic methane formation; fungi; oxygen-dependency; temperature-dependency; wood decay

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1. Introduction

Methane (CH_4) is a potent climate gas, with a greenhouse gas (GHG) potential approximately 100 times greater than carbon dioxide (CO_2) over a 10-year period [1]. A significant portion of global CH_4 emissions originates from biotic sources, exceeding contributions from abiotic sources like fossil fuel and biomass burning as well as geogenic processes [2,3].

Contrary to the earlier assumption that biotic CH_4 production occurs exclusively under anaerobic conditions by methanogenic archaea in certain environments, such as wetlands, landfills, and rice paddies, and in the digestion system of termites and ruminants, recent research has revealed that biotic CH_4 can also be produced in the presence of oxygen (O_2). The first evidence of aerobic CH_4 formation by plants was presented by Keppler et al. [4], and subsequent research expanded this finding to a range of eukaryotic CH_4 and prokaryotic sources, including mosses and lichens [5], marine algae [6,7], terrestrial and marine cyanobacteria [8], plant cell cultures [9,10], non-methanogenic archaea [10], animals [11,12], human cell cultures, and humans [10,13–16], as well as fungi [10,17,18].

For some years, the mechanisms behind CH_4 formation in these organisms remained elusive. However, a recent breakthrough came with the discovery by Ernst et al. [10] of a universal non-enzymatic CH_4 formation mechanism potentially occurring in all organisms that produce reactive oxygen species (ROS). This mechanism, based on Fenton chemistry,

involves the reaction of ROS with free iron (II) ions and methylated precursor compounds within cells, encompassing all three domains of life.

Despite these advancements, little is known about the detailed mechanism and the physical and chemical factors that drive CH_4 formation in many newly discovered sources and organisms. This is especially true for (saprotrophic) fungi that play an essential role in decomposing organic matter, such as wood lignocellulose, thus playing a crucial role in global carbon recycling [19]. The challenge lies in determining fungal biomass and correlating it with CH_4 emissions. This is further complicated by the species and medium dependency of these emissions (e.g., [18]), as well as the potential for yet unknown CH_4 formation pathways, which complicates our understanding of the global impact of fungal CH_4 emissions.

Before the studies by Lenhart et al. [17] and Schroll et al. [18], which found that saprotrophic fungi directly produce CH_4 under aerobic conditions without the presence of methanogenic archaea, it was assumed that fungi initiated the decomposition process by breaking down macromolecules, like those in wood, thereby providing the precursor compounds for CH_4 production through methanogenic archaea in anoxic microsites (e.g., [20–23]). Another CH_4-producing pathway involving facultative anaerobic fungi and a halomethane-dependent pathway was identified by Huang et al. [24], where CH_4 formation correlated with the formation of chloromethane (CH_3Cl), as previously reported by McNally et al. [25]. These authors further highlighted that the function of the enzymes involved in the halomethane-dependent CH_4 formation pathway is independent of O_2 and thus might also be involved in the observed fungal CH_4 production by Lenhart et al. [17] and Schroll et al. [18].

A common consensus is that fungal CH_4 emissions are strongly dependent on the fungal species and the wood substrates [17,18,26–28]. However, the influence of key parameters like O_2 availability and temperature on fungal CH_4 formation has not been investigated, even though these factors strongly influence the physiological activity and growth of fungi. Studies have shown that the activity and growth of xylotrophic fungi depend on prevailing O_2 mixing ratios [29–31]. For instance, xylothrophic fungi, which include the two investigated fungi in this study, can consume all available O_2 in their woody habitat and still grow under anoxic conditions [31]. On the other hand, studies have indicated that below a concentration of 0.2% O_2, fungal growth is completely inhibited [29,30], while the decay of wood debris by saprotrophic fungi decreased with decreasing O_2 and increasing CO_2 mixing ratios and vice versa [29,32]. This suggests that prevailing O_2 concentrations significantly influence fungal activity and metabolism, potentially controlling fungal CH_4 emissions.

Temperature is another critical driver of fungal metabolism. Numerous studies have investigated the relationship between wood decay and temperature [27,28,33–35], finding that increased temperatures usually lead to higher CH_4 emissions due to wood decay. This observation is likely linked to the role of saprotrophic fungi as significant producers of extracellular enzymes needed for wood decomposition, which is predicted to increase due to higher temperatures [33]. Fungal growth even quadrupled with a 10 °C increase in temperature across a tropical elevation gradient [34], indicating a substantial impact of temperature on the amount of prevailing fungal biomass and, consequently, on CH_4 emissions from xylotrophic fungi.

In our study, we investigated the effects of different O_2 levels and temperatures on fungal CH_4 production by two saprotrophic fungi, *Pleurotus sapidus* and *Laetiporus sulphureus*. Both fungal species were grown on various substrates, including beech wood, pine wood, and grass, and incubated under sterile conditions. Oxygen consumption rates were measured under different temperatures and patterns of CH_4 production, and CH_4 production normalized to O_2 consumption (CH_{4_norm}) was examined.

2. Results

In order to evaluate the dependency of fungal CH_4 formation on prevailing O_2 concentrations starting at ambient levels (20.9% O_2, see Section 2.1) and at elevated levels (starting at >90% O_2, see Section 2.2) as well as different temperature (17 to 40 °C, Section 2.3), two different saprotrophic fungal species were incubated with different growth media (beech, pine, grass). For methodological details, we refer to Section 4, Materials and Methods. Please note that CH_4 formation and O_2 consumption rates were based on a per flask basis and not related to fungal dried biomass because it was not possible to determine it after each measurement step. Thus, in all conducted incubation experiments, CH_4 production rates were normalized to the O_2 consumption rates (CH_{4_norm}) to directly link fungal CH_4 production to its metabolic activity (inferred from O_2 consumption; Table 1). In addition, changes in CO_2 concentrations in the flask were also measured and their formation rates were estimated, and these are also indicators of the metabolic activity of the fungi. As the focus of the manuscript is on the role of O_2, the accompanying CO_2 data are shown in the Supplement (Text S1 and Figures S1–S3). Please also note that all presented CH_4 formations and CH_{4_norm} rates were corrected by subtracting the observed CH_4 rates in the medium controls.

2.1. Dependance of Fungal CH_4 Production on Ambient O_2 Concentrations

All incubation experiments in which the two fungal species *P. sapidus* or *L. sulphureus* were grown on different substrates at various O_2 levels showed measurable CH_4 formation rates compared with the respective substrate control (Table 1). Formation rates of CH_4 for controls were smaller compared with fungal incubations, accounting for 0.30 ± 0.05, 0.91 ± 0.09, and 3.26 ± 0.53 nmol h^{-1} for pine wood incubated at 17 °C, 27 °C, and 40 °C, respectively, as well as 0.01 ± 0.001 and 0.31 ± 0.01 nmol h^{-1} for grass incubated at 17 °C and 40 °C, respectively. Calculated CH_4 formation and O_2 consumption rates for experiments with the two fungal species were in the range of 0 to 5.34 ± 0.64 nmol h^{-1} and 0.06 ± 0.01 to −0.88 ± 0.06 mmol h^{-1}, respectively.

No measurable CH_4 formation was observed when O_2 levels were below ~0.5%. As soon as O_2 was reintroduced, following an O_2-induced dilution of CH_4 levels in the vials, this led to an immediate increase in CH_4 formation, indicating a rapid response to the availability of O_2 for fungal metabolism. This is exemplarily shown in Figure 1 (arrows indicate the addition of O_2) for *L. sulphureus* (Figure 1A) and *P. sapidus* (Figure 1B) grown on beech wood.

For both fungi, the amount of CH_4 gradually increased within the flasks when O_2 was present. Notably, CH_4 formation rates substantially decreased in both fungi when O_2 mixing ratios fell, eventually ceasing completely, indicating no further CH_4 formation. However, upon reintroduction of O_2 to the flasks (Figure 1, as indicated by arrows, e.g., at an incubation time of 370 h for *L. sulphureus* grown on beech wood), the amount of O_2 initially increased (from 0.05 ± 0.03 to 16.8 ± 0.7 mmol) while that of CH_4 decreased (from 240.0 ± 7.8 to 190.3 ± 7 nmol) a result of the dilution effect from the supplemented gas volume. Subsequently, CH_4 formation resumed immediately leading to a consequent increase in CH_4 (~60 nmol). This pattern was repeatedly observed during the incubation of the two fungi grown on beech wood (Figure 1). It is important to note that during the incubation of *P. sapidus* grown on beech wood (Figure 1B), the CH_4 yield in the flask gradually decreased with successive O_2 additions from 167 nmol at the start of the incubation to 94.7 ± 6.0 nmol at the end of the incubation. This reduction was attributed to the dilution effect of the O_2 additions, which surpassed the fungal CH_4 formation rate. Nonetheless, a distinct increase in CH_4 was noted following each O_2 addition. Controls containing pine wood and grass (excluding beech due to unavailability, although Lenhart et al. [17] showed that beech controls show negligible CO_2 emissions) exhibited much lower but still measurable CH_4 as well as CO_2 emission rates regardless of prevailing O_2 levels (Figures S1–S3). A more detailed description and a discussion of these data can be found in the Supplement (Text S1).

Table 1. Overview of incubation experiments: fungal species, growth medium, and various temperatures (17 to 40 °C). The term "O_2 range" refers to the categories of different O_2 levels that prevailed within the incubation flasks during incubations. "N" represents the number of observations used to determine the rates of CH_4 formation, O_2 consumption, and the CH_4 formation to O_2 consumption ratio (CH_{4_norm}). These rates are presented as the arithmetic mean accompanied by the standard deviation. Please note that all rates are on a per flask basis because it was not possible to determine the dry weight of fungal biomass after each measurement step.

Fungi	Medium	Temperature [°C]	O_2 Range [%]	N	CH_4 Formation Rate [nmol h^{-1}]	O_2 Consumption Rate [mmol h^{-1}]	CH_{4_norm} [10^{-6}]
P. sapidus	beech	27	0	8	0.00 ± 0.06	0 ± 0	-
			5 to 26	38	0.48 ± 0.31	-0.57 ± 0.17	0.95 ± 0.76
	beech	27	14 to 94	4	0.61 ± 0.10	-0.88 ± 0.06	0.70 ± 0.12
	pine	17	0	3	0.04 ± 0.07	0 ± 0	-
			0 to 5	3	0.78 ± 0.43	-0.18 ± 0.04	4.18 ± 1.26
			5 to 20.9	6	0.42 ± 0.40	-0.18 ± 0.07	2.08 ± 1.23
	pine	27	0	4	0.40 ± 0.05	0 ± 0	-
			0 to 5	3	1.15 ± 0.05	-0.23 ± 0.002	4.94 ± 0.25
			5 to 20.9	4	2.12 ± 0.58	-0.53 ± 0.10	3.97 ± 0.38
	grass	17	0	2	0.05 ± 0.03	0 ± 0	-
			0 to 5	2	0.02 ± 0.01	-0.12 ± 0.03	0.19 ± 0.05
			5 to 20.9	6	0.14 ± 0.13	-0.16 ± 0.05	0.86 ± 0.62
	grass	40	0	3	0.26 ± 0.09	0 ± 0	-
			0 to 5	2	1.60 ± 0.05	-0.14 ± 0.01	11.6 ± 0.17
			5 to 20.9	7	1.41 ± 1.11	-0.43 ± 0.25	3.41 ± 1.73
L. sulphureus	beech	27	0	4	-0.02 ± 0.01	0 ± 0	-
			0 to 5	3	0.00 ± 0.03	-0.04 ± 0.002	-0.02 ± 0.70
			5 to 30.6	12	0.63 ± 0.40	-0.16 ± 0.07	3.76 ± 1.28
	beech	27	16 to 97.5	4	0.09 ± 0.02	0.06 ± 0.005	1.56 ± 0.25
	pine	17	11.1 to 20.9	3	0.15 ± 0.11	-0.03 ± 0.01	5.78 ± 5.20
	pine	27	0	3	-0.06 ± 0.05	0 ± 0	-
			0 to 5	1	2.16	-0.21	10.4
			5 to 20.9	3	5.34 ± 0.64	-0.39 ± 0.03	13.69 ± 0.97
	pine	40	0	3	-0.86 ± 0.03	0 ± 0	-
			5 to 25.8	5	1.01 ± 0.55	-0.29 ± 0.05	4.87 ± 1.49
Controls	pine	17	5 to 20.9	3	0.30 ± 0.05	-0.01 ± 0.001	35.68 ± 4.8
	pine	27	5 to 20.9	3	0.91 ± 0.09	-	-
	pine	40	0	3	1.37 ± 0.1	0 ± 0	-
			5 to 20.9	3	3.26 ± 0.53	-0.02 ± 0.004	-
	grass	17	5 to 20.9	3	0.01 ± 0.005	0 ± 0	-
	grass	40	5 to 20.9	3	0.31 ± 0.01	0 ± 0	-

Beyond the dependency of fungal CH_4 formation on the presence of O_2, we further found that the prevailing mixing ratios of O_2 in the incubation flasks influenced the CH_4 formation rates by *P. sapidus* and *L. sulphureus*. This is shown in Figure 2, where CH_4 formation as well as CH_{4_norm} rates and O_2 consumption rates (as negative values) are illustrated. While in all experiments, O_2 consumption rates were generally higher when higher O_2 mixing ratios prevailed (Table 1), this observation was more obvious for CH_4 production and CH_{4_norm} rates.

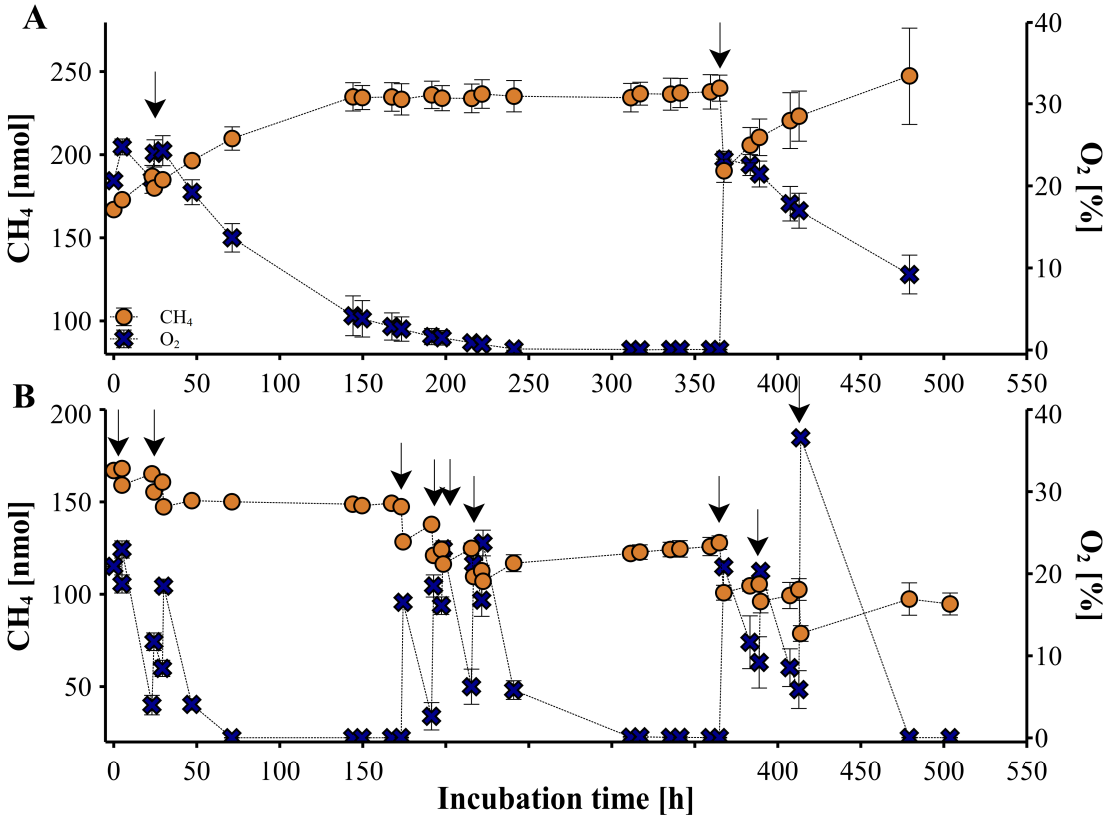


Figure 1. Amount of CH_4 and the mixing ratio of O_2 in the incubation flasks of (**A**) *L. sulphureus* and (**B**) *P. sapidus* grown on beech wood. Arrows indicate the points of O_2 addition to the individual flasks during incubation. Data points represent the arithmetic mean and standard deviation (n = 4).

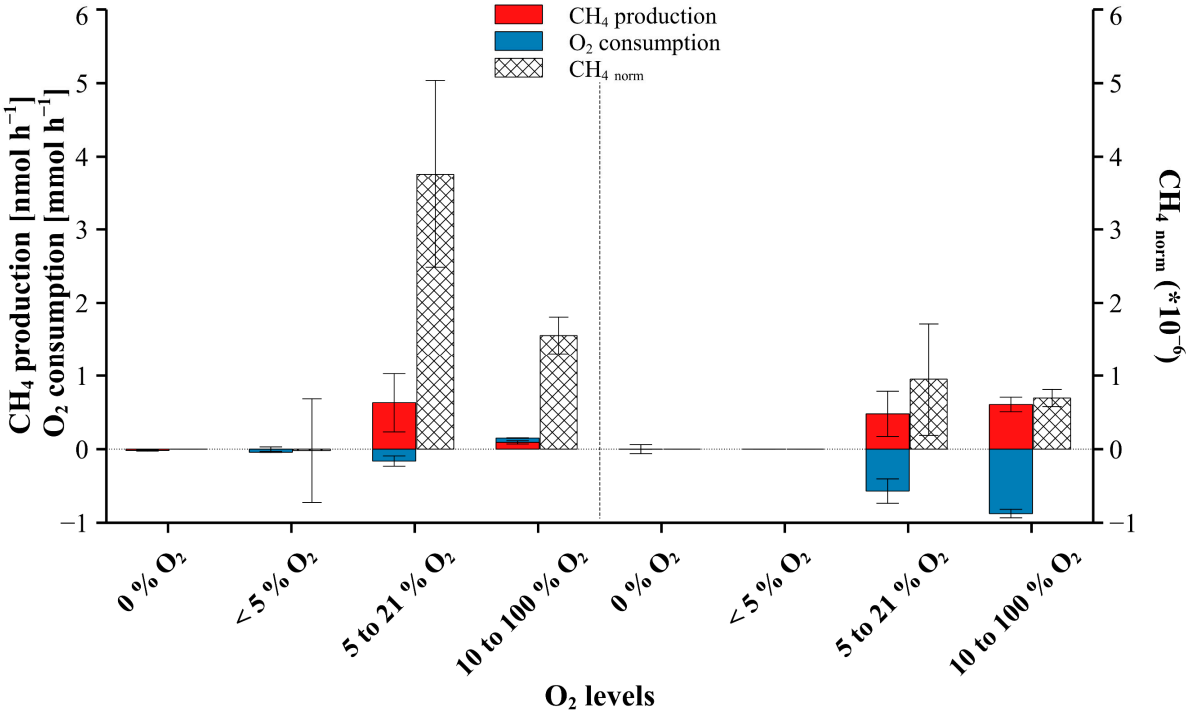


Figure 2. CH_4 production and O_2 consumption, along with CH_{4_norm} rates at different O_2 levels of *L. sulphureus* (**left panel**) and *P. sapidus* (**right panel**) grown on beech wood. The bars represent the arithmetic mean and standard deviation of observations during replicate experiments (n = 4, N = 3 to 12, see Table 1).

Generally, we observed differences between CH_4 production rates and CH_{4_norm} rates of *P. sapidus* and *L. sulphureus*. While CH_4 production rates were similar when both fungi were grown on beech wood, *L. sulphureus* showed higher rates when grown on pine wood ($p = 0.005$). Similarly, we found that CH_{4_norm} rates were generally higher for *L. sulphureus* compared with *P. sapidus* ($p < 0.001$ for beech wood and $p = 0.003$ for pine wood; Figure 2). For *P. sapidus* (except for when grown on grass at 17 °C), higher CH_{4_norm} rates were observed when O_2 levels ranged between 0 and 5% compared with higher levels between 5% and ambient (pine wood, $p = 0.057$; grass at 17 °C, $p = 0.063$; grass at 40 °C, $p < 0.001$; Table 1). For *L. sulphureus*, we observed an opposite trend, where higher CH_{4_norm} rates prevailed in a range between 5 and 21% for beech wood ($p = 0.002$) and pine wood at 27 °C (Figure 2, Table 1).

In all incubations, CO_2 mixing ratios demonstrated an opposite trend to O_2 levels, serving as a clear indicator of the fungi's metabolic activities. Please note that CO_2 measurements were conducted less frequently than those for CH_4 and O_2 due to logistical reasons. During some experiments where O_2 was added, CO_2 concentrations exceeded ~21%, a level expected when all O_2 was consumed and converted to CO_2 (Supplement Text S1 and Figures S1–S3).

2.2. Fungal CH_4 Production Starting at Elevated O_2 Mixing Ratios of Near 100%

In another approach investigating the O_2 dependency of fungal CH_4 formation, both *L. sulphureus* and *P. sapidus* were grown on beech wood at a temperature of 27 °C and exposed to a starting O_2 mixing ratio of approximately 95% by flushing the flasks with pure O_2 (Figure 3). At the beginning of the experiment, the amount of CH_4 in the incubation flasks was 27.3 ± 5.2 nmol for *L. sulphureus* and 16.9 ± 1.1 nmol for *P. sapidus* (reflecting prevailing CH_4 mixing ratios of around 0.2 and 0.4 ppmv in the flasks, respectively). These values increased to 161.3 ± 23.7 nmol (2.2 ± 0.3 ppmv) and 61.6 ± 7.1 nmol (0.8 ± 0.1 ppmv) over 1055 h and 70 h, respectively, whilst O_2 levels decreased to 16.6 ± 5.3% and 13.9 ± 3.5%. This corresponds to a CH_4 formation rate of 0.6 ± 0.1 nmol h^{-1} for *P. sapidus* and a lower rate of 0.09 ± 0.02 nmol h^{-1} for *L. sulphureus* ($p = 0.002$). Interestingly, the O_2 consumption rate varied substantially between the two fungal species. *P. sapidus* had a much higher rate accounting for −0.88 ± 0.06 mmol h^{-1}, in contrast to *L. sulphureus*, which reached a considerably lower O_2 consumption rate of −0.06 ± 0.001 mmol h^{-1} ($p < 0.001$). This disparity in O_2 consumption directly influenced the CH_{4_norm} rates, which were substantially higher for *L. sulphureus* compared with *P. sapidus*, accounting for 1.56 ± 0.25 ($\times 10^{-6}$) and 0.70 ± 0.12 ($\times 10^{-6}$), respectively ($p = 0.02$).

When comparing these CH_{4_norm} rates to the experiments starting at ambient O_2 levels of 21%, we found that CH_{4_norm} rates were in a similar range for *P. sapidus* (0.95 ± 0.76 ($\times 10^{-6}$)) and lower compared to *L. sulphureus* with 3.76 ± 1.28 ($\times 10^{-6}$).

2.3. Temperature Dependency of Fungal CH_4 Formation, O_2 Consumption, and CH_{4_norm} Rates

P. sapidus and *L. sulphureus*, grown on pine wood and grass (only *P. sapidus*), were chosen to investigate whether CH_4 formation and O_2 consumption, as well as CH_{4_norm} rates, differed based on different incubation temperatures of 17 °C, 27 °C, and 40 °C. Similarly to what is described in Sections 2.1 and 2.2, the above-mentioned rates of CH_4 formation, O_2 consumption, and CH_{4_norm} showed a clear influence of prevailing O_2 mixing ratios. This influence, along with that of different temperatures, is additionally presented in this section.

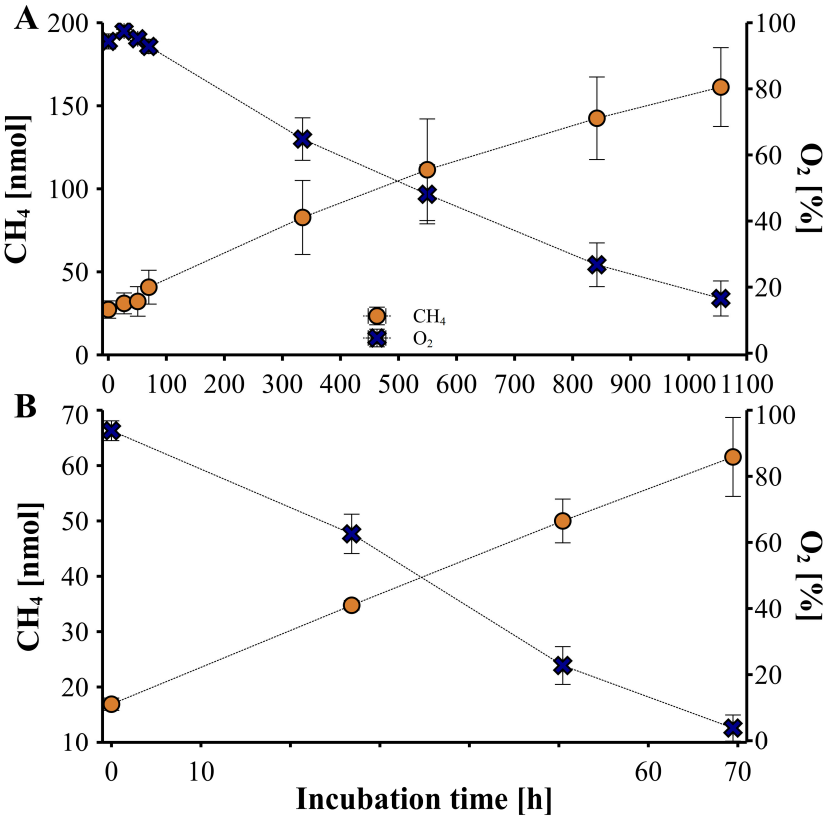


Figure 3. Amount of CH_4 and the mixing ratio of O_2 in the incubation flasks of (**A**) *L. sulphureus* and (**B**) *P. sapidus* grown on beech wood at 27 °C with incubation starting at 98% and 95% O_2, respectively. Data points represent the arithmetic mean and standard deviation of replicate experiments (n = 4).

Figure 4A,B show the trend of the amount of CH_4 and O_2 levels of the incubation experiments of *L. sulphureus* grown on pine wood at three different temperatures (17, 27, 40 °C), while Figure 4C,F show the course of these parameters of *P. sapidus* grown on pine wood (17 °C and 40 °C) and grass (17 °C and 27 °C), respectively, along with the corresponding controls for pine wood and grass. Please note that for *P. sapidus* grown on pine wood and grass, only two temperature steps were available due to logistical reasons. All three incubation experiments clearly showed that CH_4 production rates were highest at a temperature of 27 °C ($p = 0.02$ for *L. sulphureus* and $p = 0.001$ for *P. sapidus* grown on grass) or at 40 °C for *P. sapidus* grown on pine wood compared with the rates at a temperature of 17 °C ($p = 0.002$; Figure 5, Table 1). Similar observations were made for O_2 consumption rates, which were highest at a temperature of 27 °C compared with the other temperature steps. Moreover, calculated CH_{4_norm} rates at each O_2 level (see Table 1 and Figure 5) echoed this pattern. However, we observed a more complex distribution of CH_{4_norm} rates depending on the fungal species when we considered the different O_2 levels within each incubation experiment (*L. sulphureus* grown on pine and beech wood and *P. sapidus* grown on grass, pine, and beech wood; Figures 2, 4 and 5). For incubations with *L. sulphureus* grown on pine wood at 27 °C, CH_{4_norm} rates were higher for O_2 mixing ratios between 5 and 21% (13.7 ± 0.97 ($\times 10^{-6}$)) compared with rates below 5% O_2 (10.4 ($\times 10^{-6}$)). However, for *P. sapidus* grown on pine wood, CH_{4_norm} rates were higher when O_2 mixing ratios were <5% compared with O_2 levels between 5 °C and 21 °C, with rates accounting for 4.18 ± 1.26 ($\times 10^{-6}$) vs. 2.08 ± 1.23 ($\times 10^{-6}$) and 4.94 ± 0.25 ($\times 10^{-6}$) vs. 3.97 ± 0.38 ($\times 10^{-6}$) for 17 °C and 27 °C, respectively. The same observation was made for *P. sapidus* grown on grass at a temperature of 40 °C, with values of 11.6 ± 0.17 ($\times 10^{-6}$) vs. 3.41 ± 1.73 ($\times 10^{-6}$) for O_2 mixing ratios below 5% and above 5%, respectively. Contrastingly, at a temperature of 17 °C, *P. sapidus* grown on grass exhibited higher CH_{4_norm} rates when O_2 levels ranged between 5 and 21% (0.86 ± 0.62 ($\times 10^{-6}$)) as opposed to O_2 mixing ratios

below 5% (0.19 ± 0.05 ($\times 10^{-6}$)). Pine wood and grass controls showed a comparatively small increase in CH_4 levels over time depending on the temperature, with CH_4 formation rates ranging from 0.30 ± 0.05 to 3.36 ± 0.53 nmol h^{-1} for pine wood and 0.01 ± 0.001 to 0.31 ± 0.01 nmol h^{-1} for grass (Table 1, Text S2, and Figure S4).

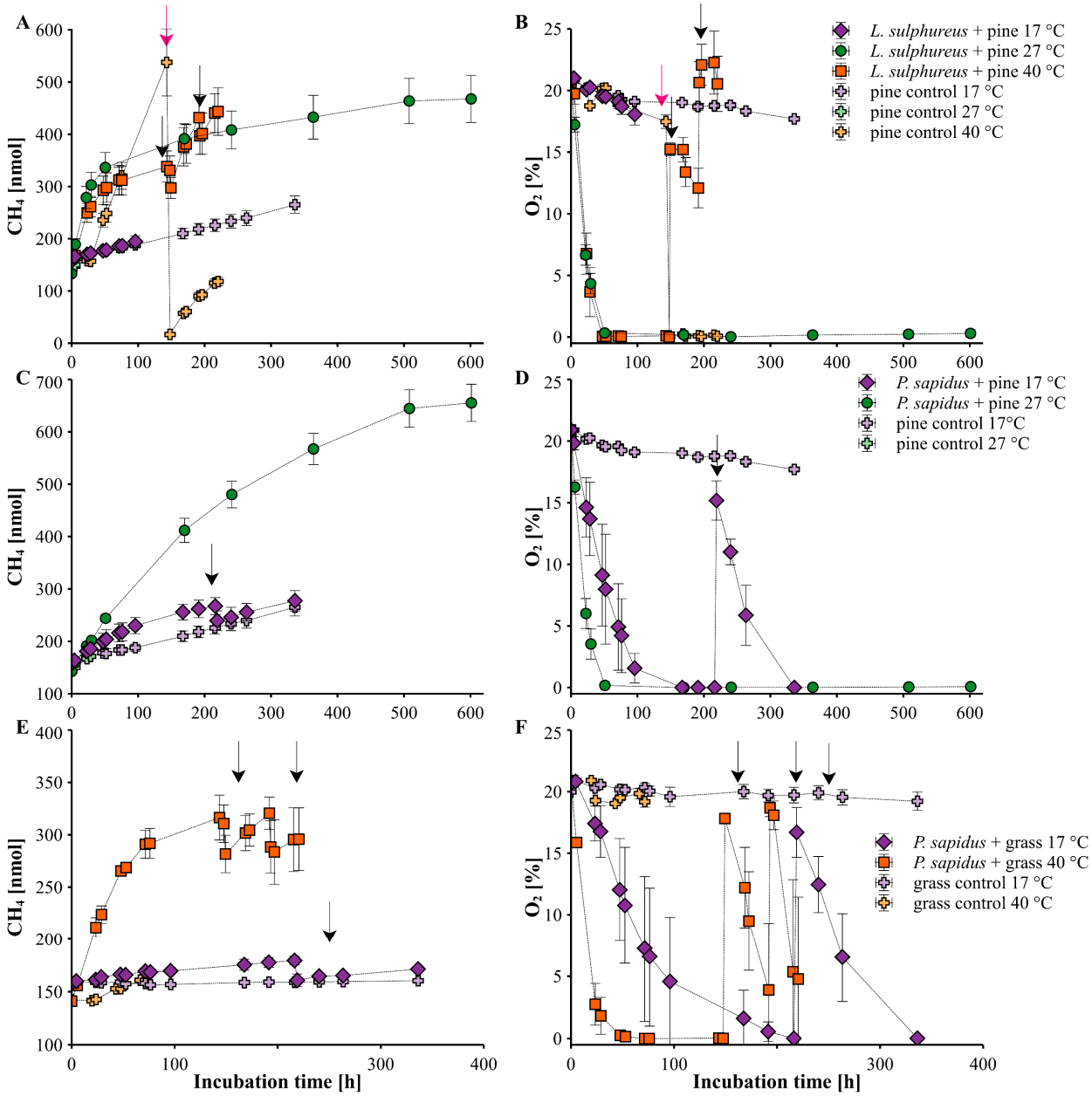


Figure 4. Changes in CH_4 amounts and O_2 levels in the flasks during incubation of (**A**,**B**) *L. sulphureus* grown on pine wood at 17, 27, and 40 °C, (**C**,**D**) *P. sapidus* grown on pine wood at 17 and 27 °C, and (**E**,**F**) *P. sapidus* grown on grass at 17 and 40 °C. Black arrows indicate the points of O_2 addition to the individual flasks containing fungi, 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).

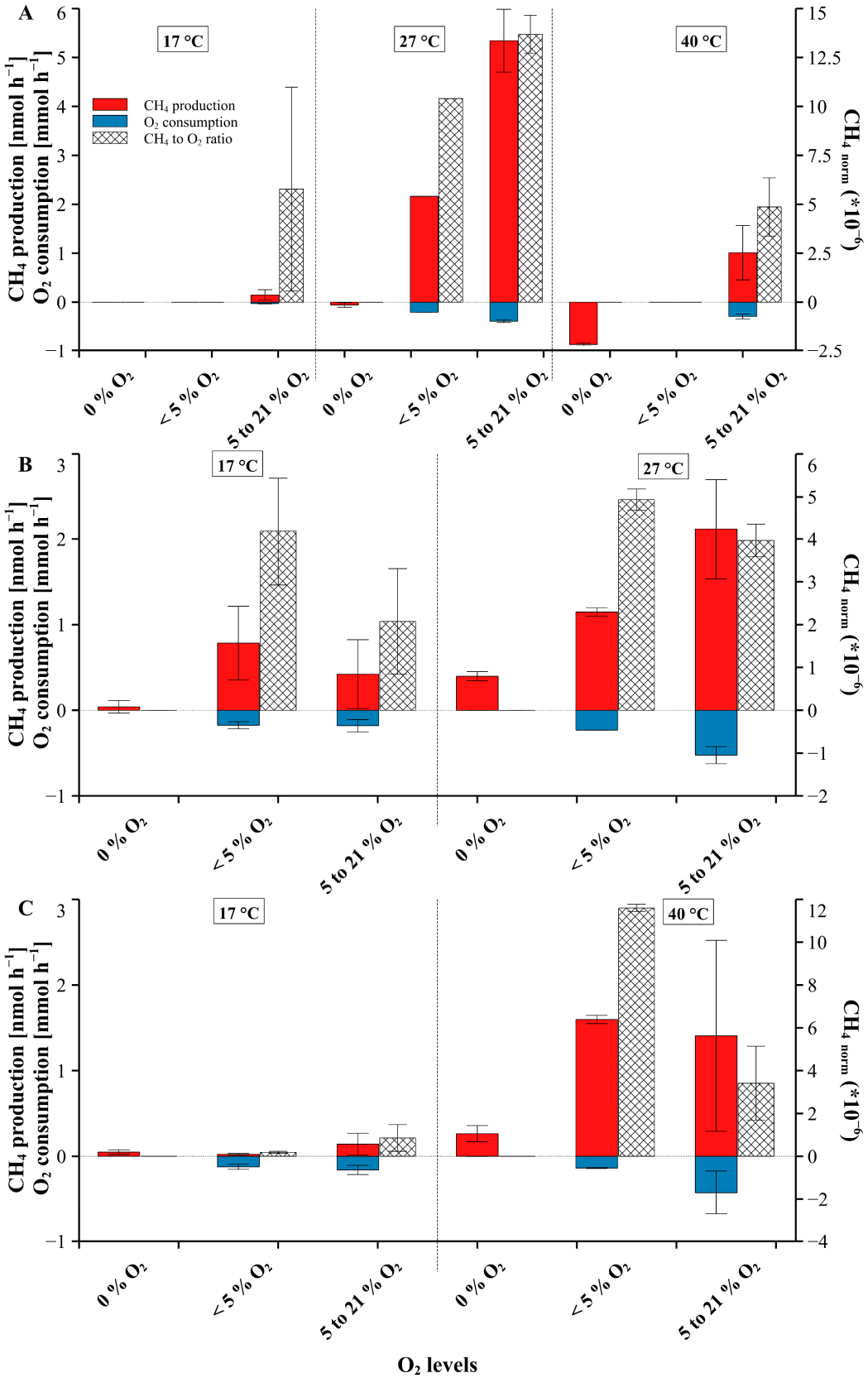


Figure 5. CH_4 production and O_2 consumption, along with CH_{4_norm} rates at varying O_2 levels (0 to 21%) and temperatures (17, 27, 40 °C), of (**A**) *L. sulphureus* grown on pine wood and (**B**) *P. sapidus* grown on pine wood and (**C**) grass. Bars represent the arithmetic mean and standard deviation of observations during replicate experiments (n = 3 to 4, N = 2 to 6, see Table 1).

3. Discussion

3.1. Dependence of Fungal CH_4 Formation on O_2 Levels

Despite existing studies on the growth, wood decay, and eco-physiological adaptations of xylotrophic fungi, the impacts of O_2 levels on CH_4 production rates and CH_{4_norm} rates have not been explored to date [36]. Our results clearly demonstrate that fungal metabolism due to the availability of O_2 is a crucial factor driving fungal CH_4 production. When O_2 was completely consumed by the fungi (meaning below ~0.5%, as indicated by the sensitivity of the deployed O_2 sensors), CH_4 formation ceased in our experiments (Figures 1 and 4). This finding contradicts earlier beliefs that linked CH_4 formation in wood debris to anoxic microsites and the activity of methanogenic archaea (Figures 1 and 4) [20–23,37]. If methanogenic archaea would have been responsible for the observed CH_4 formation in our experiments, we would have expected a strong increase in CH_4 levels once O_2 was depleted as anaerobic conditions are a prerequisite for methanogenic CH_4 formation. Instead, we observed the opposite, as no CH_4 was produced by the fungi once O_2 was depleted. This finding clearly indicates that fungal CH_4 formation is dependent on the occurrence of O_2. However, our fungal incubations were performed under sterile conditions and excluding the activity of bacteria and archaea (see Lenhart et al. [17]). Our observation is further in line with previous findings of Schroll et al. [18] that showed that δ^{13}C-CH_4 values of fungal CH_4 covers a wide range from −42 to −70 ‰, which is not exclusively indicative of methanogenic CH_4 but overlaps with many other CH_4 sources, such as thermogenic degradation of organic matter and other eukaryotes, such as algae and cyanobacteria [8,38].

Intriguingly, upon reintroduction of O_2 to the incubation flasks, inducing aerobic metabolism, O_2 consumption, and CO_2 production, fungal CH_4 formation promptly resumed (e.g., Figure 1B). Thus, our study distinctly establishes for the first time that aerobic CH_4 formation by the investigated fungal species, *P. sapidus* and *L. sulphureus*, and likely saprotrophic fungi in general, occurs exclusively for metabolically active cells in the presence of O_2. This observation is in agreement with previous results by Ernst et al. [10] showing that bacteria, such as *Bacillus subtilis,* produce CH_4 when they are active and O_2 is present. Conversely, CH_4 production by this organism halts when either the cells are in a dormant state or O_2 is absent. Thus, the pattern of CH_4 formation, dependent on O_2 levels and the metabolic activity of *B. subtilis*, appears to be a clear analogy to the two fungal species investigated in this study. Therefore, it seems likely that fungal CH_4 was produced via a mechanism similar to that described by Ernst et al. [10] via the generation of methyl radicals by oxidative demethylation of a methylated nitrogen, sulfur, or oxygen compound in the presence of ROS and iron (II) (Figure 6).

Further exploring this dependency, we discovered that the level of O_2 present in the incubation flasks significantly influenced CH_4 formation by *P. sapidus* and *L. sulphureus*. In all experiments, O_2 consumption rates were generally higher when higher O_2 mixing ratios were present. This observation aligns with prior studies indicating that O_2 levels below 0.2% strongly inhibit fungal growth [29] and O_2 levels below 10% diminish wood decay activities of saprotrophic fungi [32]. Contrarily, Mukhin and Diyarova [31] found that xylotrophic basidiomycetes could completely consume O_2 in their environment and withstand high CO_2 levels, even up to 100%. These fungi are, therefore, facultative anaerobes that produce CO_2 in O_2-deprived environments. Our observations support this, as we noticed a significant increase in CO_2 levels, even when O_2 was entirely consumed in the incubations containing medium and fungi (Figures S1–S3). It is noteworthy, however, that CO_2 mixing ratios rose more rapidly when O_2 was available to the fungi.

Our study further found that O_2 consumption, CH_4 production, and CH_{4_norm} rates differed substantially between the two fungal species, *P. sapidus* and *L. sulphureus*. The differences were most prominent in the O_2 consumption rates, where *P. sapidus* exhibited much higher rates compared with *L. sulphureus* under similar incubation conditions. This could be due to differences in biomass, however, this parameter could not be determined within the scope of this study. Another reason could be differences in their metabolic

activity, as they are different types of white rot and brown rot fungi, respectively. And, finally, the growth substrates might have impacted the observed rates. Several studies have found that fungal CH_4 production, also in relation to CO_2 emissions as an indicator of metabolic activity, is dependent on the growth substrate and substrate quality [17,18,27]. In our experiments, the most likely explanation for the observed differences is the different enzyme sets of *L. sulphureus*, as a brown rot fungus, and *P. sapidus*, as a white rot fungus.

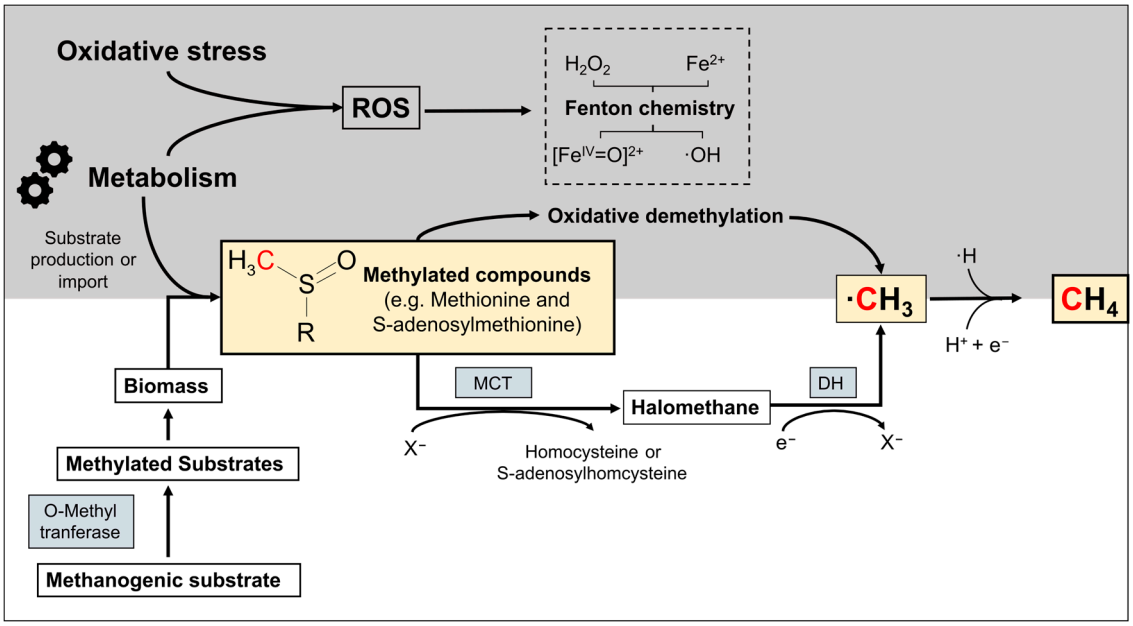


Figure 6. Overview of potential mechanisms for fungal CH_4 formation, adapted after Ernst et al. [10] (**upper part**) and Huang et al. [24] (**lower part**). The upper part illustrates the mechanisms proposed for CH_4 formation driven by reactive oxygen species (ROS) in living systems, which involves the production of methyl radicals via Fenton chemistry from methylated compounds. The lower part depicts the mechanism proposed for halomethane-mediated CH_4 formation. This process begins with a methanogenic substrate and progresses through its conversion to methylated compounds via multiple enzymatic steps (blue boxes, MCT = methyl chloride transferase, DH = dehalogenase), leading to the generation of halomethanes (X^- = Cl^-, I^-, Br^-) and, ultimately, methyl radicals.

Generally, there were notable differences in CH_4 production rates and CH_{4_norm} rates between these two fungal species. In line with previous studies by Lenhart et al. [17] and Schroll et al. [18], CH_4 production rates were typically up to 2.5 times higher in incubations containing *L. sulphureus* (grown on pine) compared with those with *P. sapidus* grown on pine (Table 1) and similar in magnitude when both fungi were grown on beech wood. Please note that for experiments where the fungi were grown on beech wood, no wood controls could be obtained. Nevertheless, previous studies by Lenhart et al. [17] clearly showed that incubation studies with sterilized beech wood controls, analogous to this study, did not increase CH_4 levels. Moreover, this observation was further validated by incubation studies in this study (see Figure 1), where no CH_4 increase was observed when O_2 was completely consumed, thus indicating that neither the fungal species nor the beech wood released CH_4 during this experiment. However, we now demonstrate for the first time that CH_{4_norm} rates normalized to O_2 consumption also exhibited a similar trend, with *L. sulphureus* showing up to 2.5 times higher values than *P. sapidus* (Figures 2 and 5).

Moreover, we observed distinct differences between *P. sapidus* and *L. sulphureus* concerning CH_{4_norm} rates under varying O_2 levels. For *P. sapidus*, CH_{4_norm} rates were often higher when O_2 levels ranged from 0 to 5%, compared with higher levels between 5% and ambient mixing ratios (Figure 5B,C; *P. sapidus* grown on pine and grass). Conversely, for *L. sulphureus*, higher CH_{4_norm} rates were observed in a range between 5 and 20%. The

underlying reason for this disparity remains unclear, but a potential explanation might lie in the differences in their metabolic pathways. *P. sapidus*, a white rot fungus, predominantly uses oxidative enzymes to decompose wood compounds, such as lignin, cellulose, and hemicellulose. In contrast, *L. sulphureus*, a brown rot fungus, relies on non-enzymatic oxidative systems to primarily depolymerize cellulose and, to a lesser extent, lignin, by generating ROS. Brown rot fungi deploy a mechanism dependent on Fenton-type reactions with ROS for wood decomposition [39–41]. However, the specific O_2 requirements for both mechanisms are not well-understood. While the O_2 requirement for brown rot fungi is relatively known due to the direct production of ROS, it is more complex for white rot fungi, where O_2 is utilized as a substrate for the enzymes associated with wood decay [40,42,43]. This aspect requires further evaluation and could be linked to the observed differences in CH_4 production and CH_{4_norm} rates between *P. sapidus* and *L. sulphureus*.

In general, the growth and wood decay of these fungi at different O_2 levels indicate that metabolic activity is closely connected to fungal CH_4 formation, given that no CH_4 is produced by either fungus when O_2 was absent. While the exact mechanism of CH_4 formation by these fungi remains elusive, initial evidence from Lenhart et al. [17], which identified methionine as a precursor of fungal CH_4, suggests that the universal CH_4 formation mechanism proposed by Ernst et al. [10] involving Fenton chemistry with methylated compounds and ROS likely represents a significant contributor to the observed formation of CH_4 (Figure 6, upper part). Nonetheless, the potential involvement of other mechanisms, such as the halomethane-dependent pathway reported by Huang et al. [24], represents another possibility (Figure 6, lower part). This study proposes a halomethane-mediated CH_4 formation mechanism in fungi, where CH_4 formation starts with a methanogenic substrate and progresses through its conversion via methylated substrates and biomass. This process leads to the formation of methylated compounds, which are then ultimately converted to halomethane and methyl radicals through multiple enzymatic steps. Although demonstrated under anaerobic conditions, the activity of the relevant enzymes for this mechanism also persists under aerobic conditions, presenting another possible CH_4 formation mechanism that warrants future investigation, particularly under aerobic conditions.

3.2. Influence of Temperature on Fungal CH_4 Formation Dynamics

To date, there is a noticeable gap in in the understanding of the influence of the temperature on fungal CH_4 formation. Previous studies have primarily focused on the growth or decomposition rates of wood via basidiomycetes, which are also critical factors for fungal CH_4 emissions. Previous studies have consistently shown that both fungal growth [34] and wood decomposition rates [27,28,33] increase with rising temperature. However, it should be noted that the fungal biomass and changes during incubation experiments could not be determined during this study, and thus CH_4 formation rates per unit biomass of fungi could not be calculated. Instead, we used consumption of O_2 and CO_2 production rates in relation to CH_4 formation as an indicator for fungal metabolic activity (see Supplement Text S1 and Figures S1–S3). We observed that the highest CH_4 formation and CH_{4_norm} rates occurred at 27 °C for both of the studied fungi. This peak in activity was likely attributed to the temperature being close to the optimal metabolism [44,45]. At temperatures both lower (17 °C) and higher (40 °C) than 27 °C, a decrease in the CH_{4_norm} rates was noted, suggesting a decline in metabolic activity, as further indicated by lower O_2 consumption and CO_2 production rates.

It is important to acknowledge that higher fungal biomass resulting from elevated growth rates most likely leads to increased CH_4 formation. This is due to CH_4 being produced by the fungus itself, as reported by Ernst et al. [10]. This study found that two fungal species produced CH_4 and that elevated levels of ROS, which can increase in organisms as a stress response, even amplified the observed CH_4 formation. This aligns with findings from Lenhart et al. [17] and Schroll et al. [18], which showed fungal CH_4 formation independent of the presence of methanogenic archaea. Therefore, it appears that

CH_4 formation may be a function of not only the fungal biomass and experienced stress (ROS) levels but also the metabolic activity of the fungal species.

At 17 °C, *P. sapidus* grown on grass exhibited smaller CH_4 production rates compared with when it was grown on pine wood, despite similar O_2 consumption rates across different O_2 regimes. Consequently, CH_{4_norm} rates were much higher for *P. sapidus* grown on pine wood. This indicates a substrate-specific component regulating CH_4 formation rates, supporting previous studies that highlighted the strong effect of fungal substrate on CH_4 formation and decomposition rates [18,27]. Additionally, CH_4 production rates for *P. sapidus* grown on grass and pine wood increased at elevated temperatures of 40 °C and 27 °C, respectively, likely due to higher metabolic activity, as indicated by increased O_2 consumption rates, particularly when O_2 mixing ratios exceeded 5%. This observation could also be influenced by increased stress levels, especially for *P. sapidus* grown on grass at 40 °C, in a similar way as the previously discussed higher CH_4 formation in fungi due to increased ROS [10].

A similar pattern was noted for *L. sulphureus* grown on pine wood at 17 °C, 27 °C, and 40 °C. The highest CH_4 production rates were found at 27 °C, which is presumably closest to the optimal fungal growth temperature, whereas the lowest CH_4 formation rates at 17 °C coincided with the lowest O_2 consumption rates, indicating reduced metabolic activity at this temperature compared to 27 °C and 40 °C. However, contrastingly, CH_{4_norm} rates were in a similar range for both 17 °C and 40 °C, suggesting that while metabolic activity (inferred from O_2 consumption) was higher at 40 °C, the ratio of CH_4 formation to O_2 consumption (CH_{4_norm}) remained in the same range of around 3.5 (10^{-6}). Mukhortova et al. [28] also observed that CH_4 and CO_2 levels clearly increased with temperature, ranging from 5 °C to 25 °C, during the decomposition of woody debris in a northern boreal forest, indicating an aerobic mechanism for CH_4 formation as reported by [10,17,18], which opposes the notion of an exclusive anaerobic origin of CH_4 through methanogenic archaea in anoxic microsites of these woody debris.

At various temperatures, we found that without O_2, no or substantially less CH_4 was emitted during our incubation experiments. Notably, CH_4 formation observed in the controls was also temperature-dependent, suggesting an additional abiotic mechanism, as previously suggested by Lenhart et al. and Schroll et al. [17,18]. For the results of the control experiments and explanations of abiotic CH_4 formation, we refer to the Supplement Text S2 and Figure S4. Regarding the lack of knowledge about abiotic formation of CH_4 and CO_2 from the studied substrates, substantially more research is required to fully comprehend this phenomenon.

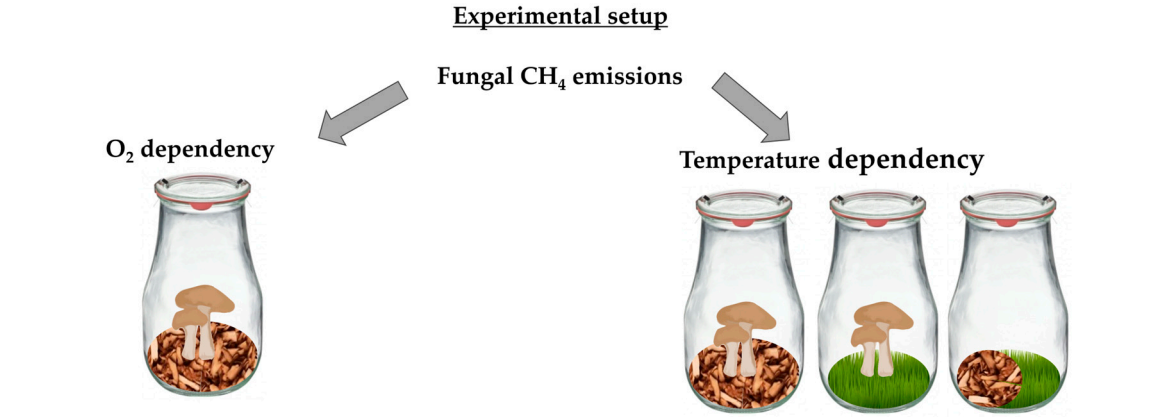
4. Materials and Methods

4.1. Selected Fungi

In this study, we selected *P. sapidus* (Pleurotaceae, DSMZ 8266) and *L. sulphureus* (Polyporacaeae, DSMZ 1014) due to their documented CH_4 emission properties, as reported in previous studies by Lenhart et al. and Schroll et al. [17,18]. These organisms were specifically chosen not only for their CH_4 emission capabilities but also for their distinct ecological and physiological traits. *P. sapidus*, a white rot fungus, and *L. sulphureus*, a brown rot fungus, are both notable for their ease of cultivation and management in laboratory settings, making them ideal species for our experimental analysis.

4.2. Incubation Experiments

We investigated the O_2 and temperature dependency of fungal CH_4 emissions, focusing on the two fungal species *P. sapidus* and *L. sulphureus*. *P. sapidus* was cultivated on a variety of media, including beech, pine, and a grass mixture, whereas *L. sulphureus* was grown on beech wood and pine wood. The cultivation process involved using autoclaved wood chips from these trees and the grass mixture, sterilized at 121 °C and 2 bar pressure for 20 min (Figure 7).



Fungi	*L. sulphureus*	*P. Sapidus*	
Medium	beech wood		
O_2 levels [%]	0 to 30.6	0 to 26.0	Experiment 1
	16.6 to 97.5	14.0 to 94.5	Experiment 2
Temperature [°C]	27		
n	4	4	

Fungi	*L. Sulphureus*			*P. sapidus*				Controls	
Medium	pine wood			pine wood		grass		pine wood	grass
Temperature [°C]	17	27	40	17	27	17	40	17, 27, 40	17, 40
O_2 levels [%]	11. 1 to 20.9	0 to 20.9	0 to 25.8	0 to 20.9				20.9	
n	3	4	4	3	4	3	3	3 each	

Figure 7. Experimental setup for investigating the O_2 and temperature dependency of fungal CH_4 formation. Fungal CH_4 formation was examined through concentration measurements in incubation flasks containing fungi *L. sulphureus* or *P. sapidus* grown on beech wood, pine wood, or grass under varying O_2 levels (**left column**) and incubation temperatures of 17 °C, 27 °C, or 40 °C (**right column**). "n" represents the number of replicate experiments.

These sterilized media (~350 g) were then placed into 2.7 L glass flasks (Weck, Bonn, Germany) and inoculated with pure fungal submerged cultures (50 mL), maintaining sterile conditions as described by Lenhart et al. [17]. In parallel, controls of the medium were prepared in a similar way, except for the inoculation with fungal cultures. To facilitate gas exchange, each flask was sealed with a rubber band and a glass lid, which incorporated a hole plugged with a cotton stopper. Before the beginning of the incubation period, the flasks were aerated under sterile conditions to establish the initial atmospheric ratios of CH_4, O_2, and CO_2. Subsequently, the cotton stoppers were replaced with sterile silicone stoppers (Saint-Gobain Performance Plastics, Courbevoie, France) to limit uncontrolled gas exchange while allowing for precise gas sampling.

In order to investigate the temperature dependency of fungal CH_4 emissions, the fungi were placed in a climate chamber set at three different temperatures: 17 °C, 27 °C, and 40 °C. The duration of incubation varied, extending up to ~1100 h (ca. 46 days).

Gas samplings were performed as follows. First, the pressure within the glass flasks was measured using an EX- portable pressure-measuring instrument (GHM Messtechnik GmbH, Regenstauf, Germany) with a precision of ±1%. Then, a plastic syringe (Plastikpak, NJ, USA) was used to extract 10 mL of gas for CH_4 measurements and 6 mL for CO_2. Measurement of CH_4 and CO_2 mixing ratios was performed according to the procedures detailed in Section 4.3, with atmospheric air replacing the extracted gas. Alongside gas sampling, we continuously monitored O_2 concentrations within the flasks using calibrated O_2 spots (Section 4.3).

Furthermore, to maintain specific O_2 mixing ratios and to test the hypothesis of the O_2 dependency of fungal CH_4 emissions, especially in cases where O_2 was depleted or fell below certain concentrations, we regulated the O_2 levels in the flasks by adding pure O_2 until the desired concentration (usually to atmospheric O_2 levels), which was immediately controlled using the calibrated O_2 spots. For the pine wood control incubated at 40 °C, CH_4 formation was, in addition to ambient O_2 mixing ratios, investigated without the presence of O_2 by exchanging the whole headspace volume with pure helium.

4.3. Measurement of CH_4, CO_2, and O_2 Concentrations

Mixing ratios of CH_4 in this study were measured using a gas chromatograph coupled with a flame ionization detector (GC-FID; Shimadzu 14b, Kyoto, Japan). To facilitate this, sample gas was injected into the GC-FID through a six-port valve (Valco Instruments, Houston, TX, USA), which was linked to a chemical trap filled with Drierite®. This setup was crucial for drying the gas before entering the analytical system via a sample loop with a volume of 2 mL that was employed using a 20 mL plastic syringe (Plastipak BD, Franklin Lakes, NJ, USA). The GC itself was equipped with a stainless-steel column (3.175 mm in inner diameter) packed with a 60–80-mesh molecular sieve 5A (Supelco, St. Louis, MO, USA), effectively separating CH_4 from other gas components in the samples. The oven temperature was maintained constantly at 125 °C. To quantify CH_4 within the samples, daily measurements (ranging from 3 to 4) of two reference standards with CH_4 mixing ratios of 2.192 and 9.655 ppmv were conducted.

For measuring CO_2 mixing ratios, a GC coupled with a barrier discharge ionization detector (BID; Shimadzu, Kyoto, Japan) was employed. Here, 50 µL of sample gas was injected into the GC-BID via an autosampler AOC-20-i (Shimadzu, Kyoto, Japan) using a split injection method (5:1). This GC was equipped with a ShinCarbon ST packed column (80/100 mesh; length: 2 m; diameter: 0.53 mm; Shimadzu, Kyoto, Japan). The quantification of CO_2 was achieved by measuring various reference standards (400 ppm, 0.5%, 10%, and 40% by volume each conducted in triplicate). As part of quality control, one reference standard was measured after every 6 to 9 single measurements.

Oxygen concentrations were determined using non-invasive optical sensors (O_2 spots; PSt3 sensor type) and a Fibox 4 portable measuring instrument (both from PreSens Precision Sensing GmbH, Regensburg, Germany). These O_2 spots were installed in the glass flasks before the start of the incubation experiments and calibrated using a two-point calibration with ambient air (20.9% O_2) and helium (0% O_2). The precision of the O_2 spots is 0.4% at 20.9% O_2 and 0.05% at 0.2% O_2.

4.4. Calculations and Statistical Methods

In this study, all CH_4 emission rates were carefully normalized against the respective O_2 consumption rates to ensure the investigation of CH_4 formation based on the fungal metabolism. The CH_4 formation rates, based on O_2 levels, were categorized into three distinct groups: 0% O_2, 0–5% O_2, as well as >5 to 21% O_2 (for experiments with fungi grown on pine wood and grass) and >5 to 98% (for experiments with fungi grown on beech wood). This categorization was pivotal, as it was observed that below an O_2 mixing ratio of 5%, there was a noticeable reduction in CH_4 production.

To systematically categorize the normalized CH_4 rates within these defined O_2 levels for each flask, sections corresponding to the respective O_2 mixing ratios were manually defined. Within these sections, CH_4 emission and O_2 consumption rates were calculated using linear regression analysis for all incubation experiments. Importantly, respective CH_4 formation rates of the controls were subtracted from the calculated rates in the control of the respective substrate.

For each incubation experiment, arithmetic means and standard deviations were calculated to discern differences between the treatments. Furthermore, t-tests were conducted to provide statistical backing for the observations made. However, it is important to note that this study adhered to the recommendations of the American Statistical Association. Consequently, *p*-values and other statistical parameters were not solely relied upon as the criteria for drawing conclusions [46]. Thus, the term "statistically significant" was consciously avoided in the interpretation of the results.

5. Conclusions

This study, to our knowledge, is the first to investigate how different O_2 mixing ratios and temperatures control CH_4 emissions in two saprotrophic fungi, *L. sulphureus* and *P. sapidus*. We observed that CH_4 formation rates are highly dependent on the prevailing

O_2 mixing ratios. Notably, in all of our incubation experiments, we observed that fungal CH_4 formation rates diminished when O_2 mixing ratios fell below approximately 0.5%. Conversely, CH_4 formation increased immediately after O_2 was reintroduced or when O_2 levels remained above this threshold, unambiguously highlighting the role of aerobic metabolism for fungal CH_4 formation. Furthermore, we found that CH_{4_norm} rates varied based on the fungal species and their substrates, including beech wood, pine wood, and grass. These findings suggest that the investigated fungal species, and possibly also further fungal species, produce CH_4, and that aerobic metabolism controlled by O_2 levels is a critical factor in this process. This further challenges the previous assumption that in the fungal realm, CH_4 is only formed under anoxic conditions and that saprotrophic fungi simply provide methanogenic archaea with precursor compounds via the decomposition of woody components. Additionally, our study shows that temperature has a substantial effect on fungal CH_4 formation. We observed lower CH_4 formation and CH_4_norm rates at temperatures of 17 °C and 40 °C, while the highest rates of CH_4 formation occurred at 27 °C, indicating that this temperature is closer to the optimum metabolic activity for the investigated fungi.

Thus, our research clearly demonstrates that both the availability of O_2 and temperature are key in controlling fungal CH_4 emissions. From an environmental perspective, temperature increases due to climate change could significantly increase fungal CH_4 emissions through enhanced fungal growth and biomass. Therefore, understanding CH_4 formation and its controlling factors, especially temperature and O_2 levels, is crucial for assessing the role of fungal CH_4 emissions regarding global CH_4 fluxes. Additionally, exploring these fungal CH_4 dynamics in various environments, such as forests, soils, and aquatic systems, ref. [47] under both aerobic and anaerobic conditions is necessary to better understand their impact on carbon and greenhouse gas dynamics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/methane3020015/s1, Text S1: Oxygen and temperature dependency of CO_2 mixing ratios during fungal incubations; Figure S1: Changes in 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 in 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 in 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 S4: Changes in CH_4 amounts as well as O_2 and CO_2 levels in the flasks during control incubation of pine wood at 17, 27, and 40 °C and 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). Refs. [48–56] have been cited in the Supplementary Material.

Author Contributions: M.S.: conceptualization, methodology, formal analysis, investigation, writing—original draft, writing—review and editing, visualization. K.L.: conceptualization, methodology, resources, writing—review and editing, supervision, funding acquisition. T.B.: formal analysis, investigation. P.H.: formal analysis, investigation. A.R.: formal analysis, investigation. S.S.: formal analysis, investigation. F.K.: conceptualization, methodology, resources, writing—review and editing, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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