Faculty of Biosciences, Fishery, and Economics

Life on Air: On the Physiological Basis of Atmospheric Methane Oxidizing Bacteria

Tilman Schmider

A dissertation for the degree of Philosophiae Doctor, March 2024

Table of Contents

3

Acknowledgements

Before I was allowed to work in the laboratories of UiT, I had to join a course about health, safety, environmental matters, and emergency preparedness. During this course, the lecture stated that "*my leader is the most important person in my life*" which is, especially for a German, a delicate statement. Of course, he was referring to my PI and Supervisor Alex. Even though I do not fully agree with the statement, amongst others, because my partner in life Andrea might read the acknowledgement, there is definitely a grain of truth if referred to the work life of a PhD candidate. When I first met Alex during my masters in Vienna, thinking I was talking to another master student (guess the fresh ocean air does not only conserve cod), I had no idea of his plans to recruit Andrea as postdoc for his research group in Tromsø. But with Andrea as arctic soil enthusiast and me as good and planless partner, it was quickly settled that I would end up in Tromsø. Luckily, I did not regret the decision at all. Alex, you did a great job not only scientifically but also socially as PI and supervisor, which, unfortunately, does not seem to be the default in science. Many thanks for that!

My original PhD topic addressed the methanotrophic community of a pingo system in Svalbard. As the pandemic rendered the sampling on Svalbard impossible, I was forced to change the PhD topic and focus on methanotrophic pure cultures that appear to grow on air. This change significantly minimized the risk of drowning in a dark, muddy pond or being assimilated by a polar bear. Also, it gave me the opportunity to continue the research on these fascinating bacteria, that was started by the group under Mette's leadership. Thus, Mette and Alex, many thanks that you enabled this unique opportunity.

Even though these fascinating bacteria I focused on can grow on air, they want to be wined and dined. Due to their special nature they demand to be grown on filters when fed with air which requires a lot of work. As Anne Grethe prepared most of the countless filter cultures that I have burned during my PhD and helped me a lot in general, a big thanks to you Anne Grethe.

If one starts to work at a new workplace, it is always helpful to have a person around that knows the lab, the infrastructure, and the right people. It is especially helpful if the person is as nice and willing to help as Bente. Bente, thank you for your help whenever it was needed.

Next, I want to thank my office mate Yin, all members of the CECO and the Microplants group which I am part of, and all co-authors contributing to the publications. It is nice to work in an environment mainly driven by curiosity as major motivation. You made my time as PhD really enjoyable!

Natürlich will ich auch meiner Familie, die mich in meinem bisherigen Werdegang immer unterstütz hat, danken. Phrasen wie diese erscheinen mir immer etwas leer, darum versuche ich es auf den Punkt zu bringen: Eintauschen will ich euch nicht.

And of course, I want to thank Andrea. I guess the lecturer confused Alex with you.

For those that I have not mentioned by name or forgotten, even though they have earned my thanks, many thanks and please excuse me. I am writing this section at the very end of my thesis. Most likely, your name was sacrificed to the submission deadline.

Abstract

Atmospheric methane oxidizing bacteria (atmMOB) constitute the only known biological sink for atmospheric methane (CH_4). By oxidizing the potent greenhouse gas CH_4 at its atmospheric concentration, they mitigate global warming. Despite the discovery of atmMOB in soils worldwide, the research on atmMOB stagnated as methanotrophs, that oxidize atmospheric CH_4 enduringly, have escaped isolation for decades. In 2019, the isolation of an atmMOB *Methylocapsa gorgona*, capable of growing "on air" (i.e., with air as sole carbon and energy source), eventually succeeded. Building on this atmMOB isolate, the physiological strategies and metabolic traits enabling methanotrophic growth on air have been investigated during this PhD thesis. To do so, a set of methods, specifically designed for bacterial cultures growing on filters that float on medium, has been established, comprising trace gas oxidation experiments, cell quantification, comparative proteomics, and ^{15}N-based isotopic labelling. Besides *M. gorgona*, the capability of five additional methanotrophic species to grow on air and their physiological strategies were investigated. In total, four of the six methanotrophic species, including three outside the canonical atmMOB group USCα, could grow on air, quadrupling the number of known atmMOB in pure culture. Additionally, the atmMOB were found to cover their nitrogen requirements from air. A combination of three physiological characteristics was identified as basis of the atmMOB to grow on air: A mixotrophic lifestyle, as all species oxidized atmospheric hydrogen (H_2) and/or carbon monoxide (CO) in addition to CH_4; energy requirements for growth below the energy value previously assumed necessary for cellular maintenance; and high apparent specific affinities for CH_4. On a metabolic level, the atmMOB decreased investments in biosynthesis while increasing investments in trace gas oxidation during growth on air. As a first comprehensive work on atmMOB in pure culture, this thesis reveals strategies of atmMOB to grow on air, challenging previous notions of energy constraints for aerobic growth and the phylogeny of atmMOB. The findings suggest that the atmosphere supports larger atmMOB populations than assumed, indicating that their global distribution and impact on the global carbon cycle have been underestimated. Also, their role in mitigating global warming might be more important as they consume the indirect greenhouse gases H_2 and CO in addition to CH_4. The atmMOB pure cultures and the methods developed in this thesis, are promising steppingstones to model the interaction between rising atmospheric CH_4 levels, global warming, and the biological sink for atmospheric CH_4, and to evaluate the potential of atmMOB for the biofiltration of low-CH_4 emissions and other biotechnological applications.

List of papers

Paper 1:

Simultaneous Oxidation of Atmospheric Methane, Carbon Monoxide and Hydrogen for Bacterial Growth

Alexander Tøsdal Tveit, Tilman Schmider, Anne Grethe Hestnes, Matteus Lindgren, Alena Didriksen and Mette Marianne

Paper 2:

Physiological Basis for Atmospheric Methane Oxidation and Methanotrophic Growth on Air

Tilman Schmider, Anne Grethe Hestnes, Julia Brzykcy, Hannes Schmidt, Arno Schintlmeister, Benjamin R. K. Roller, Ezequiel Jesús Teran, Andrea Söllinger, Oliver Schmidt, Martin F. Polz, Andreas Richter, Mette M. Svenning, Alexander T. Tveit

Abbreviations

a_A^0	–	specific apparent affinity
ANME	–	anaerobic methane oxidizing archaea
AtmMOB	–	atmospheric methane oxidizing bacteria
ATP	–	adenosine triphosphate
c	–	gas concentration
CH$_2$O	–	formaldehyde
CH$_3$OH	–	methanol
CH$_4$	–	methane
C-mol	–	carbon mol
CO	–	carbon monoxide
CO$_2$	–	carbon dioxide
CODH	–	carbon monoxide dehydrogenase
D$_2$O	–	deuterium oxide/heavy water
EMP	–	Earth Microbiome Project
Fae	–	formaldehyde-activating enzyme
Fch	–	formate-tetrahydrofolate ligase
Fdh	–	formate dehydrogenase
FolD	–	methylene-H$_4$F dehydrogenase/methylene-H$_4$F cyclohydrolase
GCS	–	glycine cleavage system
H$_2$O	–	water
H$_4$F	–	tetrahydrofolate
H$_4$MPT	–	tetrahydromethanopterin
HCOO$^-$	–	formate
Hhy	–	NiFe group 1h hydrogenase
kJ	–	kilojoule
K$_m$	–	half saturation constant
K$_{m(app)}$	–	apparent half saturation constant
LEL	–	lower explosive level
MDH	–	methanol dehydrogenase
MMO	–	methane monooxygenase
MOB	–	aerobic methane oxidizing bacteria
MtdA	–	methylene-H$_4$F/ methylene-H$_4$MPT dehydrogenase
MtdB	–	methylene-H$_4$MPT dehydrogenase
N$_2$	–	dinitrogen
NanoSIMS	–	secondary ion mass spectrometry
NH$_3$	–	ammonia
Nif	–	molybdenum-iron nitrogenase
O$_2$	–	dioxgen
OAA	–	oxaloacetate
OH	–	hydroxyl radicals
PBS	–	phosphate saline buffer
PHB	–	poly-β-hydroxybutyrate
pMMO	–	particulate methane monooxygenase
ppb	–	parts per billion
ppm	–	parts per million

PPQ	–	pyrroloquinoline quinone
sMMO	–	soluble methane monooxygenase
SMR	–	suspended microchannel resonator
USCα	–	upland soil cluster alpha
USCγ	–	upland soil cluster gamma
v	–	oxidation rate
V$_{max}$	–	maximal oxidation rate
V$_{max(app)}$	–	apparent maximal oxidation rate

1 Introduction

1.1 Atmospheric methane

Despite low atmospheric concentrations, methane (CH_4) is a greenhouse gas with a strong impact on climate change. Over a time horizon of 100 years, its global warming potential is estimated to be 30 times higher than carbon dioxide (CO_2). On a 20-year timescale, the global warming potential of CH_4 surpasses the potential of CO_2 80 times[1,2]. This time dependent change in the global warming potential of CH_4 derives from its short atmospheric lifetime of ca. 12 years[3]. CH_4 is responsible for about 20% of the global warming induced by long-lived greenhouse gases. Since pre-industrial times, the atmospheric concentration of CH_4 has increased by 150% to 1911.81 ± 0.59 parts per billion (ppb) in 2022[1,4,5] (Fig.1).
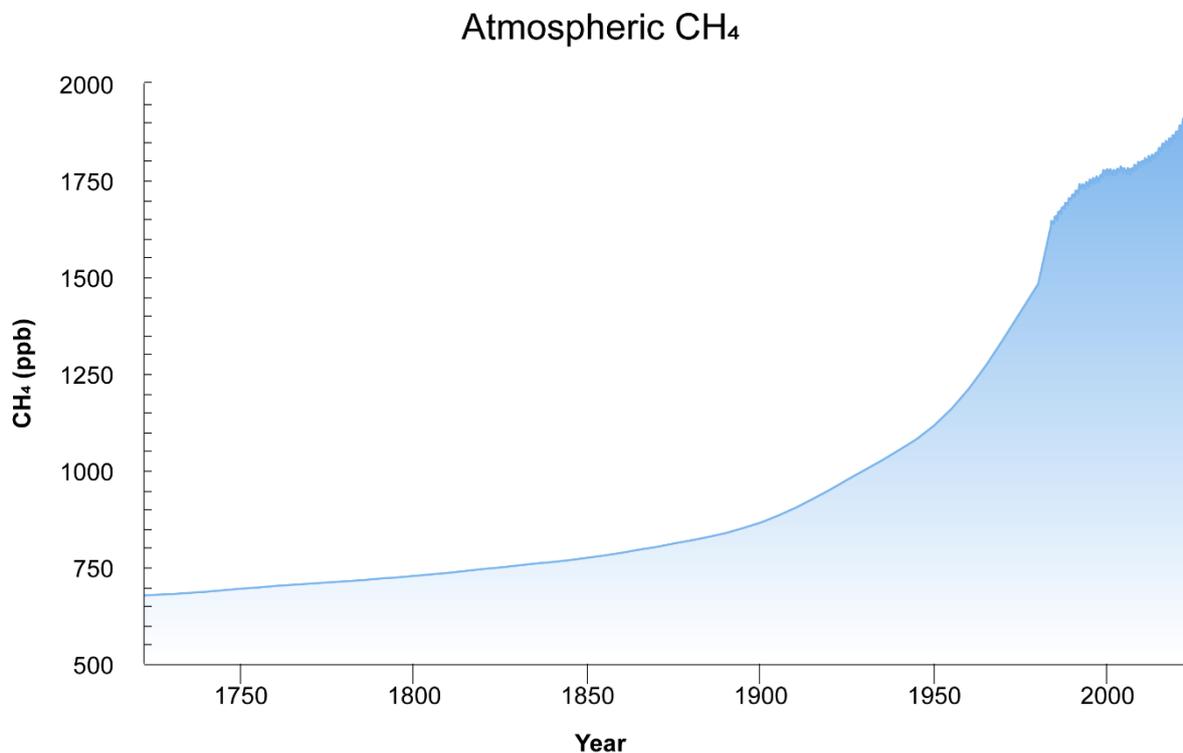
Figure 1. Atmospheric CH_4 concentration in ppb since 1750 (https://www.methanelevels.org/).

CH_4 sources that cause the increasing atmospheric concentrations can be broadly categorized into pyrogenic, thermogenic, and biogenic groups[6]. Pyrogenic CH_4 originates from the partial combustion of fossil fuels and biofuels and of biomass and soil carbon during wildfires. Thermogenic CH_4 has been formed over millions of years through geological processes. It seeps from the subsurface to the atmosphere via mud volcanos, marine seeps, terrestrial seeps or

through exploitation of fossil fuels[6]. For a long time, strictly anaerobic microorganisms belonging to the domain of Archaea (methanogens) have been considered as the only considerable biogenic CH_4 source. However, a study demonstrated the generation of CH_4 by aquatic and terrestrial cyanobacteria under light, dark, oxic, and anoxic conditions, linking CH_4 production to phototrophic primary production[7]. In 2022, a study reported CH_4 formation in metabolic active organism triggered by free iron and reactive oxygen species, implying that all living cells possess the potential to generate CH_4[8]. Still, the major sources of biogenic CH_4 are inhabited by methanogens and comprise a wide range of anoxic anthropogenic and natural ecosystems, ranging from rice paddies, digestive systems of ruminants, organic waste deposits, and oxygen-poor freshwater reservoirs to wetlands, oceans, and termites[6,7]. The major sink of atmospheric CH_4 is the chemical oxidation of CH_4 by hydroxyl radicals (OH) in troposphere and stratosphere accounting for approximately 95% of the loss[6,9,10]. The remaining 5% of the estimated loss is caused by methanotrophic bacteria that constitute the sole biological sink of atmospheric CH_4.

1.2   Methanotrophs

Methanotrophs, that utilize CH_4 as energy and carbon source, are the only known biological CH_4 sink[11,12]. They comprise a phylogenetically diverse group of anaerobic and aerobic prokaryotes that oxidize CH_4 to CO_2 and play a major role in the global CH_4 cycle as they mitigate CH_4 emissions before reaching the atmosphere. It is estimated that methanotrophs mitigate 500 – 798 Tg CH_4 per year[9,13]. In anoxic environments, anaerobic CH_4 oxidizing archaea (ANME) oxidize CH_4 by coupling the oxidation to the reduction of the electron acceptors sulfate, nitrate, iron, or manganese. In addition, methanotrophic bacteria of the candidate phylum NC10 oxidize CH_4 in anoxic environments using an intra-aerobic pathway that generates oxygen (O_2) from nitrite[14–18]. In oxic environments, aerobic methane oxidizing bacteria (MOB) oxidize CH_4 with O_2 to CO_2. Atmospheric methane oxidizing bacteria (atmMOB), a subgroup of MOB, constitute the above-mentioned biological sink for atmospheric CH_4 as they can oxidize CH_4 at its atmospheric concentration. The defining characteristic of MOB is the methane monooxygenase (MMO) that catalyzes the oxidation of CH_4 to methanol. Depending on their major pathway of carbon fixation, aerobic methanotrophs have been divided into three groups: Type I methanotrophs, mainly dominated by

Gammaproteobacteria, fix carbon via the ribulose monophosphate pathway. Type II methanotrophs use the serine pathway for carbon fixation and belong to the class of *Alphaproteobacteria*[19]. Some Type II methanotrophs within the family *Beijerinckiaceae* also encode the Calvin-Benson-Bassham Cycle although the functional role of the cycle in these methanotrophs remains unclear[20]. Type III methanotrophs fix carbon via the Calvin-Benson-Bassham Cycle and belong to the *Verrucomicrobia*[21]. However, the finding of methanotrophs within the *Actinobacteria* that fix carbon also via the ribulose monophosphate pathway renders the classification based on carbon fixation pathways as outdated[22]. Additionally, a recent study suggests that aerobic methanotrophs might also be represented within the *Gemmatimonadota*[23]. Thus, the classification of aerobic methanotrophs into *types* should be only considered as synonym for the phylogenetic groups[19] and needs to be updated according to the latest scientific progress.

1.3 Why study atmMOB and the biological sink of atmospheric CH$_4$?

Since 2007, after a brief period of stability, the atmospheric CH$_4$ concentration started to increase rapidly[3] (Fig. 1). This increase has been linked to several causes: The declining OH concentration in the atmosphere that weakens the major atmospheric CH$_4$ sink, the increase of anthropogenic CH$_4$ emissions from agricultural and fossil fuel sources, and the increasing microbial CH$_4$ production in wetlands. The increasing microbial production suggests that current increases in the atmospheric CH$_4$ concentration are also driven by microbial feedback responses to global warming[24–26]. In contrast to the declining chemical sink for atmospheric CH$_4$, the biological sink has the potential to grow with increasing concentrations of atmospheric CH$_4$. As the biological sink is within reach of management practices, its natural potential might be maximized to increase the removal of atmospheric CH$_4$[27]. Additionally, atmMOB might be promising biocatalyst for the biofiltration of CH$_4$ emissions[28]. However, the ecology, phylogeny, and especially the physiology of atmMOB is subject to major uncertainties caused by a historical lack of atmMOB in pure culture. These uncertainties have therefore prevented efficient management of the biological sink; understanding and predicting feedback responses by atmMOB to increasing concentrations of atmospheric CH$_4$; and the utilization of atmMOB for the biofiltration of emissions containing CH$_4$. Thus, a better understanding of atmMOB

might, in light of the climate crisis, offer a toolset to mitigate CH_4 emissions, convert mitigated CH_4 emissions efficiently into valuable carbon compounds, and cope with increasing atmospheric concentrations of CH_4.

1.4 Atmospheric methane oxidizing bacteria

Despite the discovery of aerobic methanotrophy by Kaserer and Söhngen in the early 20th century, the first indication of atmospheric CH_4 oxidation was reported in 1982[29]. In that study, swamp soils were shown to emit CH_4 when waterlogged, but consume atmospheric CH_4 when dry. The discovery of biphasic CH_4 oxidation kinetics of soils by Bender and Conrad in 1992[30], was another early indication of the existence of atmMOB. They discovered that soils exposed to low and high CH_4 concentrations show different kinetic properties. At low CH_4 concentrations, they measured high affinity activities and low maximal methane oxidation rates (V_{max}). Exposing the same soil to high CH_4 concentrations led to low affinity activities and high V_{max}. They concluded that methanotrophs only cultivatable at high CH_4 concentrations (in literature also referred to as canonical or conventional methanotrophs) are responsible for the low affinity activity. The high affinity, however, was assigned to either an unknown group of methanotrophs or an activity of the "canonical" methanotrophs that had escaped discovery[31]. Either way, as the investigated soils, in contrast to autoclaved controls, showed oxidation activity at CH_4 mixing ratios of 1700 ppb, another evidence was supplied that methanotrophic bacteria can act as sink for atmospheric CH_4. Since the discovery of the biphasic CH_4 oxidation kinetics, two major, yet partially unanswered questions have shaped the research in the scientific field: Which methanotrophs are responsible for the oxidation of atmospheric CH_4 and how can these methanotrophs survive and grow with the apparent energy limitations inherent to the oxidation of the low atmospheric CH_4 concentrations?

1.5 Ecology and phylogeny of atmospheric methane oxidizing bacteria

Atmospheric CH_4 oxidation has been mainly linked to aerobic and dry soils that are often referred to as "upland soils". In these soils, atmMOB oxidize CH_4 that diffuses from the overlying atmosphere into the soil[32]. Examples for soil ecosystems in which net uptake of

atmospheric CH_4 has been measured are manifold, including forest soils[33,34], tropical soils[35], grasslands and meadows[36], landfill cover soils[37], deserts[38], heathlands[39], dryland rice soils[40,41], tundra soils[42,43], and dry swamp soils[29]. However, also caves[44] and the surfaces of plants like birches, spruces[45], and mosses[46] can represent sinks for atmospheric CH_4. Studies, that coupled the *pmoA* gene of MOB (encodes the β subunit of the particulate methane monooxygenase) as functional marker to isotope or fluorescence labeling, assigned the oxidation of atmospheric CH_4 in upland soils to the activity of two clades within the *Alpha-* and *Gammaproteobacteria*, the upland soil cluster alpha (USCα) and the upland soil cluster gamma (USCγ)[47–49]. Besides the abundance of USCα and USCγ, the *pmoA* of representatives within *Methylocystaceae*, *Beijerinckaceae*, *Methylococcaceae*, *Crenotrichaceae* and cluster 1 – 5, have been found in environments with net CH_4 uptake[33,50,51].

As summarized by Dunfield[32] and Kolb[33], different environmental factors affect methanotrophic communities and the biological sink for atmospheric CH_4 in soils. These factors comprise, among others, temperature, diffusivity, water content, pH, nitrogen availability, and anthropogenic disturbances. It is important to keep in mind that these factors should not only be considered isolated but that their interactions modify their individual impact[52,53].

King et al. concluded from CH_4 oxidation rates of forest soils and methanotrophic communities at different temperatures and different CH_4 concentrations, that temperature has a rather weak effect on the uptake of atmospheric CH_4 by the methanotrophic community[54]. According to the study, this low response to temperature changes by methanotrophs is caused by the limited diffusion of CH_4 into the soil at atmospheric CH_4 concentrations as methanotrophs exposed to high CH_4 concentrations showed a strong response to temperature change[54]. The diffusion limitation at atmospheric CH_4 concentrations is also reflected by a gradient of decreasing CH_4 observed in soils (e.g., Whalen et al. 1992[55]) as rapid diffusion of CH_4 from the atmosphere should not result in a gradient. A study modelling the uptake of atmospheric methane by soils between the years 1900 and 2100, also identified diffusion as a major factor driving atmospheric CH_4 oxidation but in addition pointed out that temperature became an important factor for the increasing methanotrophic rates in soils[56]. Increasing temperature can stimulate or suppress CH_4 oxidation as it influences the enzyme activity[52]. However, the effect of temperature on growth, the physiological state of atmMOB cells, and the CH_4 oxidation rate has never been studied directly as isolates of atmMOB were unavailable. Besides direct impacts on CH_4

oxidation, temperature can change soil moisture, and thus cause water stress that decreases CH_4 oxidation or improve gas diffusion that increases CH_4 oxidation[57]. The diffusion of CH_4 in water is 10 000 times slower than in air[32,55]. Thus, the water content is a critical factor impacting CH_4 diffusion, CH_4 availability, and eventually oxidation rates by atmMOB at atmospheric CH_4 concentrations.

The pH does not seem to have a large effect on the oxidation of atmospheric CH_4 by soils as oxidation has been detected in wide range of soils with different pH[33]. However, major shifts in pH can lead to a loss of CH_4 oxidation activity[33,58]. Furthermore, pH is a main driver of the methanotrophic community composition in environments representing atmospheric CH_4 sinks as it influences the abundance of USCα and USCγ[33,47,50,59,60]. USCα members are more likely to be abundant in acidic environments while members of the USCγ are abundant in alkaline environments, a trend also observed for "conventional" methanotrophs within the *Alpha-* and *Gammaproteobacteria*[61].

Since the beginning of industrialization, the anthropogenic release of reactive nitrogen has more than doubled the nitrogen input into global ecosystems[62]. The increased input is mainly caused by fertilizer usage, combustion of fossil fuels, and industrial activities. Reactive nitrogen is also emitted to the atmosphere causing an increased nitrogen deposition to surfaces and thus impacts terrestrial and marine ecosystems globally by modifying the biogeochemical nitrogen cycle[63,64]. Amongst others, consequences of nitrogen deposition on ecosystems are fertilization, acidification, and accumulation of excess nutrients[63,65]. How the increased nitrogen input and availability of reactive nitrogen in general influences the biological sink for atmospheric CH_4 sink is unclear[66]. Often, nitrogen fertilization inhibits atmospheric CH_4 oxidation[67,68], but also stimulatory effects of nitrogen fertilization have been reported[69,70]. Overall, the impact of nitrogen on CH_4 oxidation seems to depend on the diversity and activity of methanotrophic community, the type and amount of the reactive nitrogen, and the exposure time to the nitrogen fertilizer[69,71,72]. In contrast to the extensively studied effect of increased reactive nitrogen on the biological sink for atmospheric CH_4, the effect of nitrogen limitation on atmMOB and the sink is unknown as a lack of atmMOB in pure culture has prevented research[66]. Besides changes in the nitrogen input, other anthropogenic activities impact the biological sink for atmospheric CH_4[73]. For example, the conversion of ecosystems to agricultural sites disturbs the uptake of atmospheric CH_4 as agriculture practices can, among others, cause limitation of gas diffusion,

16

water stress, soil compaction, disruption of the soil structure, and increased bulk density[73–75]. Additionally, agrochemicals, logging, and soil acidification can decrease atmospheric CH$_4$ oxidation[76–79].

1.6   Physiology of atmospheric methane oxidizing bacteria

Dunfield[32] summarized four potential strategies of atmMOB that might enable survival and growth with the apparent energy limitations inherent to the oxidation of the low atmospheric CH$_4$ concentrations in air:

(i)   atmMOB are oligotrophs that manage to grow and survive on atmospheric CH$_4$ alone.

(ii)   atmMOB are flush feeders that feed on CH$_4$ generated in deeper soil layers in addition to atmospheric CH$_4$.

(iii)   atmMOB grow on substrates in addition to atmospheric CH$_4$.

(iv)   atmMOB grow on other substrates than atmospheric CH$_4$ and the CH$_4$ is co-oxidized in a process that does not yield energy.

1.6.1   (i) Oligotrophs

Conrad reviewed[80] the theoretical constrains for microorganisms oxidizing CH$_4$, hydrogen (H$_2$), and carbon monoxide (CO) as substrate for their energy metabolism. Traces of all three gases are present in the atmosphere at mixing ratios of 1919 ppb for CH$_4$ (August 2023 https://gml.noaa.gov/ccgg/trends_ch4/), 530 ppb for H$_2$, and 90 ppb for CO[81,82]. The Gibbs free energy resulting from the oxidation of mentioned gases at atmospheric concentrations add up to -797 kJ mol^{-1} (assuming a CH$_4$ concentration of 1750 ppb), -199 kJ mol^{-1}, and -235 kJ, respectively. Therefore, the low atmospheric partial pressure of the gases does not constrain microorganisms thermodynamically. Rather, according to Conrad[80], the constraints are of kinetic nature. The oxidation of low gas concentration needs to proceed quick enough to generate sufficient energy for cellular maintenance and growth. Assuming Michaelis-Menten kinetics, this can be explained based on the following equation:

$$v = c \times \frac{V_{\max(app)}}{K_{m(app)}} \quad (I)$$

In which v is the oxidation rate, c the gas concentration, $V_{\max(app)}$ the apparent maximal oxidation rate of the cell, and $K_{m(app)}$ the apparent half saturation constant of the cell. In general, the half saturation constant (K_m) (half saturation constant for an enzyme) displays the gas concentration at which the enzymes involved in the oxidation processes are half-saturated. Hence, the constant provides information about binding affinity of enzyme and substrate. The higher the binding affinity, the tighter the enzyme binds the substrate. The K_m is defined as dissociation constant and thus, a low K_m translates into a high affinity and vice versa. On a cellular level the apparent affinity $K_{m(app)}$ (half saturation constant of a cell) needs to be coupled to a sufficiently high $V_{\max(app)}$ (which can be achieved by a high number of enzymes per cell) to meet cellular maintenance energy requirements at atmospheric gas concentrations. Thus, the apparent specific affinity that puts $V_{\max(app)}$ and $K_{m(app)}$ into relation seems to be a valid measure of oligotrophy, while the high apparent affinity as measure is ambiguous since it doesn't give information about the oxidation rate and eventually the corresponding energy yield (Fig.2). The apparent specific affinity is defined as:

$$a_A^0 = \frac{V_{\max(app)}}{K_{m(app)}} \quad (II)$$

and represents the initial slope of a Michaelis-Menten hyperbolic curve and the pseudo first-order rate constant[83]. It directly indicates reaction rates at low substrate concentrations catalyzed by microorganisms[84]. Since the oxidation rate v equals the product of ambient gas concentration c and specific affinity a_A^0 (equation I), a high apparent specific affinity is necessary to meet cellular maintenance energy requirements at atmospheric CH_4 concentrations. In a study by Tijhuis et al.[85], stating that maintenance energy is mainly influenced by temperature, the average microbial maintenance energy is estimated to be 4.5 kJ per C-mol biomass per hour at 25° C. The value is in so far important as it is the basic premise for later theoretical assumptions on how methanotrophs live on atmospheric CH_4 concentrations by Conrad[80].
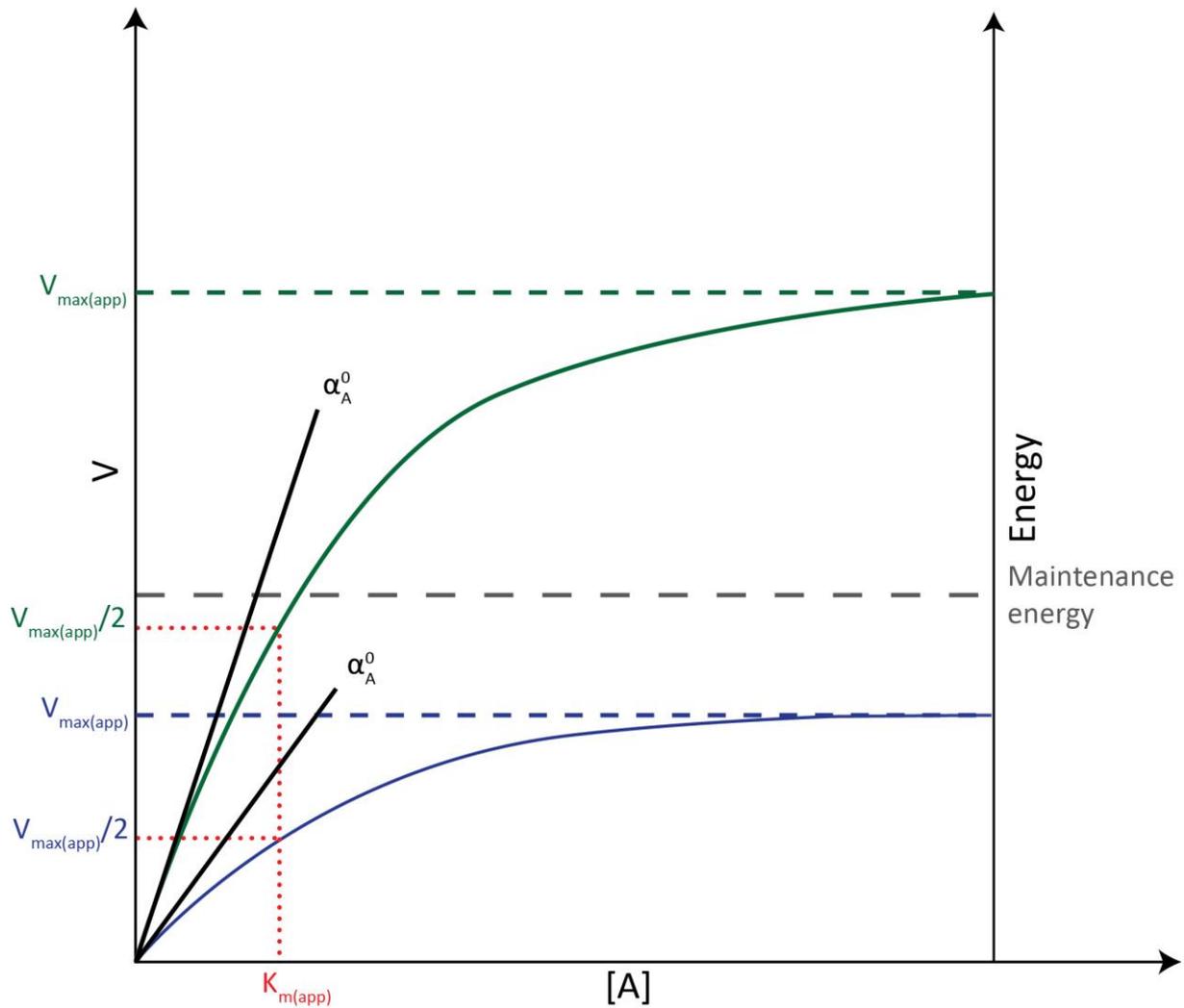
Figure 2: Two different Michaelis Menten hyperbolic curves with the same $K_{m(app)}$ but different $V_{max(app)}$ to illustrate a_A^0 and the related energy yield. [A] substrate concentration (e.g. CH_4). The $K_{m(app)}$ is defined as the substrate concentration at which $V_{max(app)}$ is half ($V_{max(app)}/2$).

The lack of pure cultures has prevented evaluation of the theory that atmMOB are oligotrophs capable of growing on atmospheric CH_4 alone. However, the theory that atmMOB are oligotrophs capable of growing on atmospheric CH_4 alone is supported by low apparent half saturation constants ($K_{m(app)}$) < 0.1 µM found in upland soils (Fig. 3).

19
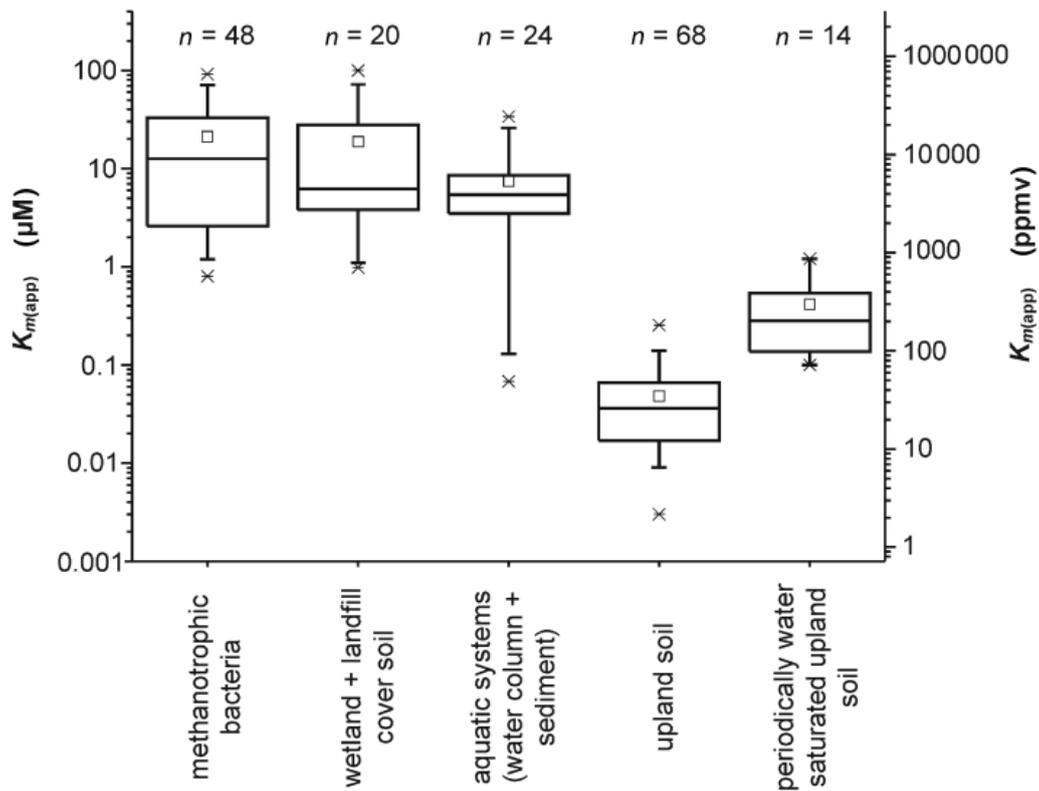
Figure 3. Box plot of $K_{m(app)}$ values for methane oxidation in pure cultures of methanotrophic bacteria and in different ecosystems. The upper and lower box lines represent the 25th and 75th percentile values (= 50% of all values). The horizontal line in the box represents the 50th percentile (median) and the square symbol the mean. Error bars denote the 5th and 95th percentiles and asterisks the upper and lower limits[86].

Until now, the only energy yield estimations for atmMOB at atmospheric CH_4 are derived from environmental studies. For example, a study[87] estimated the CH_4 oxidation rate by the MOB community in forest soils partly dominated by USCα. There, the rate was assessed by combining qPCR-based quantification of MOB and their CH_4 oxidation, amounting $540 - 800$ x 10^{-18} mol CH_4 cell^{-1} hour^{-1}. The value is more than 10 times higher than the oxidation rate needed (40 x 10^{-18} mol CH_4 cell^{-1} hour^{-1}) to supply the above-mentioned maintenance energy (4.5 kJ per C-mol biomass per hour). Thus, the study supports the theory that atmMOB are oligotrophs, i.e., being able to survive and grow on atmospheric CH_4 alone. However, the authors revoked this energy estimation in later study stating that the qPCR-based approach likely overestimated the cell-specific CH_4 oxidation rates. In the later study, the authors reported atmospheric CH_4 oxidation rates of $1 - 14$ x 10^{-18} mol CH_4 cell^{-1} hour^{-1} in forests soils[88].

1.6.2 (ii) Flush feeders

The second theoretical strategy described by Dunfield[32] implies that atmMOBs are flush feeders. They feed on CH_4 periodically generated in deeper soil layers in addition to atmospheric CH_4. The CH_4 concentration in soil layers can be elevated during periods with increased soil water content (e.g. after rainfall) as the high soil water content restricts O_2 diffusion. The decreased O_2 tension creates suitable conditions for methanogens to generate CH_4. The resulting increased CH_4 concentration stimulates "conventional" methanotrophs to oxidize CH_4. After the CH_4 concentration has decreased to an atmospheric level, methanotrophs oxidize atmospheric CH_4 until they transition into an inactive state. Indications of flush feeding were, for example, observed in alpine tundra soils that were first exposed to H_2 and CO_2 to induce hydrogenotrophic CH_4 generation[88]. After the CH_4 concentration fell back to atmospheric, the atmospheric CH_4 oxidation rates of these soils were three times higher than of soils serving as controls. A study targeting the methanotrophic community in paddy soils provided first evidence for flush feeding as "conventional" methanotrophs abundant in the paddy soils regained the ability to oxidize atmospheric CH_4 for several days after being exposed to high CH_4 concentrations[89]. Additionally, it was shown that a *Methylocystis* strain oxidized atmospheric CH_4 for three months after being cultivated under a 10% CH_4 atmosphere[90]. A potential process underlying flush feeding is the accumulation of storage compounds that allows MOB to stay active for a certain period of starvation. Presumably, during exposure to increased CH_4 concentrations, methanotrophs build up storage compounds such as poly-β-hydroxybutyrate (PHB) or glycogen. These storage compounds can later be used as reducing equivalents and are together with the oxidation of atmospheric CH_4 sufficient to meet cellular growth and maintenance requirements. Several studies reported storage compound accumulation in methanotrophs and determined different reasons for the accumulation. PHB can be accumulated if reducing energy is excessive[91], but also induced stress and nutrient- and nitrogen-limitation seem to trigger PHB accumulation[89,92]. Pure culture-based studies reported PHB accumulation ranging between 7 – 68% of the total dry weight in *Methylocystis* and *Methylosinus* cells[92,93]. In line with that, a recent study on the gammaproteobacterial methanotroph *Methylobacter tundripaludum* SV96 demonstrated accumulation of glycogen at high temperatures[94]. While these data demonstrate that storage compounds are utilized by methanotrophs, there is still no direct evidence that such compounds play a role in the growth of MOB at atmospheric CH_4 concentrations.

1.6.3 (iii) Facultative CH$_4$ oxidizers

The third theory on how atmMOB grow, assumes that they are facultative methanotrophs, i.e. in addition to atmospheric CH$_4$, the organisms can utilize other substrates[32]. By showing that "conventional" methanotrophic *Methyllocella* species can grow on the multicarbon compounds acetate, pyruvate, succinate, malate, and ethanol, the conjecture that the single carbon intermediates of aerobic CH$_4$ oxidation, methanol, formaldehyde, and formate, are the only potential energy source, was proven wrong[95]. Additionally, members of the genera *Methylocystis* and *Methylocapsa* were shown to grow on acetate and/or ethanol[96–99]. Further, a representative of the genus *Methylocystis* has been shown to oxidize H$_2$ in addition to CH$_4$ as energy source[100]. *Methylocapsa* gorgona MG08, central to this thesis as it represents the first methanotroph in pure culture found to grow with air as the only substrate, encodes not only a NiFe group 1h hydrogenase (Hhy) but also a MuCo class 1 carbon monoxide dehydrogenase (CODH)[101]. Thus, it carries the potential to gain additional energy through the oxidation of H$_2$ and carbon monoxide (CO). The same study revealed the genomic potential to use these gases in several other members of the genera *Methylocapsa*, *Methylocystis*, and *Methylosinus*. However, whether the oxidation of mentioned gases at atmospheric trace concentrations contribute to the energy conservation by atmMOB remained unclear at that point.

1.6.4 (iv) Ammonia oxidizers

The last theory proposed by Dunfield[32] to explain atmospheric CH$_4$ oxidation is based on ammonia-oxidizing bacteria. The key enzyme of ammonia oxidation, the ammonia monooxygenase, and the methane monoxygenase are very likely to share the same evolutionary origin[102,103]. The two enzymes have been found to oxidize ammonia (NH$_3$) and CH$_4$ as both compounds are structurally analogous. However, there is no proof that the resulting toxic methanol can be further utilized by ammonia oxidizers[104]. Nonetheless, ammonia oxidizers might contribute to atmospheric CH$_4$ oxidation by co-oxidizing CH$_4$ instead of NH$_3$, but their impact on atmospheric CH$_4$ oxidation seems to be neglectable[32].

1.7   Main objectives

My PhD thesis builds up on the study "Widespread soil bacterium that oxidizes atmospheric methane" by Tveit et al.[101] that was published in 2019. The study reports the isolation and an initial characterization of *Methylocapsa gorgona* MG08, the first known atmMOB able to grow with air as sole energy and carbon source, and the first member of the USCα in pure culture. The lack of atmMOB in pure culture has prevented the assessment of the above-mentioned potential strategies that enable growth on atmospheric CH_4 concentrations for many years. By finally having atmMOB in pure culture, my PhD thesis aimed at deciphering the physiological strategies of *M. gorgona* MG08 that enable its growth on air. Additionally, in growth tests prior to my project, it has been shown that not only *M. gorgona* MG08, but also five additional methanotrophic strains within the three different genera *Methylocapsa*, *Methylocystis*, and *Methylosinus*, all belonging to *Alphaproteobacteria*, form colonies when cultivated on filters floating on medium and with air as the only source of energy and carbon. These five strains are *Methylocapsa acidiphila* B2, *Methylocapsa aurea* KYG, *Methylocapsa palsarum* NE2, *Methylocystis rosea* SV97, and *Methylosinus trichosporium* OB3b. These strains, as well as *M. gorgona* MG08, encode functional complexes for the oxidation of H_2 and/or CO (Fig. 4) and thus the potential to oxidize these two gases in addition to CH_4. The physiology underlying colony formation of these methanotrophic strains is likely based on one or more of the three theoretical strategies described in detail in the previous chapter (see section 1.6): (i) AtmMOB as "Oligotrophs" that grow with methane as the sole carbon and energy source; (ii) AtmMOB as "Flush feeders"; (iii) AtmMOB as "Facultative oxidizers" of additional atmospheric gases. By investigating the six different methanotrophic strains and their physiological strategies to live on air, the PhD aimed to answer the following questions which represent my main objectives:

- What is the physiological basis for atmospheric CH_4 oxidation?
- Do the methanotrophic strains oxidize the atmospheric trace gases H_2 and CO in addition to CH_4?
- What is the energy yield per cell during oxidation of trace gases in air?
- Do the metabolic strategies of atmMOB vary between strains?
- Is atmospheric CH_4 oxidation geographically and phylogenetically more widespread than previously thought?

1.8   Secondary objective

In addition to my main objectives a secondary objective is based on the fact that the six strains encode all genes required for dinitrogen (N_2) fixation[101] (Fig. 4) and show growth on nitrogen-free medium[101,105–109] when cultivated at high concentrations of CH_4 implying N_2 fixation by the strains. Additionally, growth experiments with four of the MOB strains cultivated on filters while being exposed to air as sole substrate and nitrogen source resulted in colony formation indicating that the strains might be able to fix N_2 when grown with air as their only source of carbon, energy, and nitrogen. If true, the N_2 fixation could provide a crucial advantage for life in oligotrophic soils and atmMOB would not only play an important role in the carbon cycle as atmospheric CH_4 sink but also impact the nitrogen cycle and serve as potential nitrogen source in nitrogen limited environments. However, N_2 fixation is presumably too costly[110] for atmMOB if the theoretical energy assumptions by Conrad[80] hold true. In addition to the energy costs for N_2-fixation, the nitrogenase, the key enzyme involved in the N_2 fixation process, is very O_2 sensitive. Given the fact that some of the mentioned strains only seem to fixate N_2 under reduced O_2 tension, N_2-fixation of the strains while being exposed to air seems to be rather unlikely. However, based on the observation of colony formation on nitrogen-free medium and air as sole substrate, the PhD targeted the following question representing my secondary objective:

- Do the atmMOB strains fix N_2 during growth with air as sole carbon, energy, and nitrogen source?

1.9   The methanotrophic strains

The following section comprises a description of the methanotrophic strains that were screened for their ability to grow with air as sole carbon and energy source during this PhD thesis.

1.9.1   *Methylocystaceae*

Two of the strains that show colony formation when grown on filters exposed to air, namely *Methylosinus trichosporium* OB3b and *Methylocystis rosea* SV97, belong to the family *Methylocystaceae* within the class *Alphaproteobacteria*. As reviewed by Dedysh and Knief[19], methanotrophs within this family of gram-negative bacteria fix carbon via the serine pathway.

Methylosinus trichosporium OB3b, one of the most extensively studied methanotrophs, is an exospore forming, mesophilic, motile, rod-shaped microorganism with the capability to fixate N_2[105,111]. Depending on the copper availability it expresses either soluble methane monooxygenases (sMMO) or particulate methane monooxygenases (pMMO) (see section 1.10 below). Under N-limited growth conditions, the cells can accumulate up to 55,5% PHB[112,113]. Besides the functional complex for pMMO and a nitrogenase, it also encodes all subunits of a Hhy hydrogenase and an incomplete CODH complex[101] (Fig. 4).
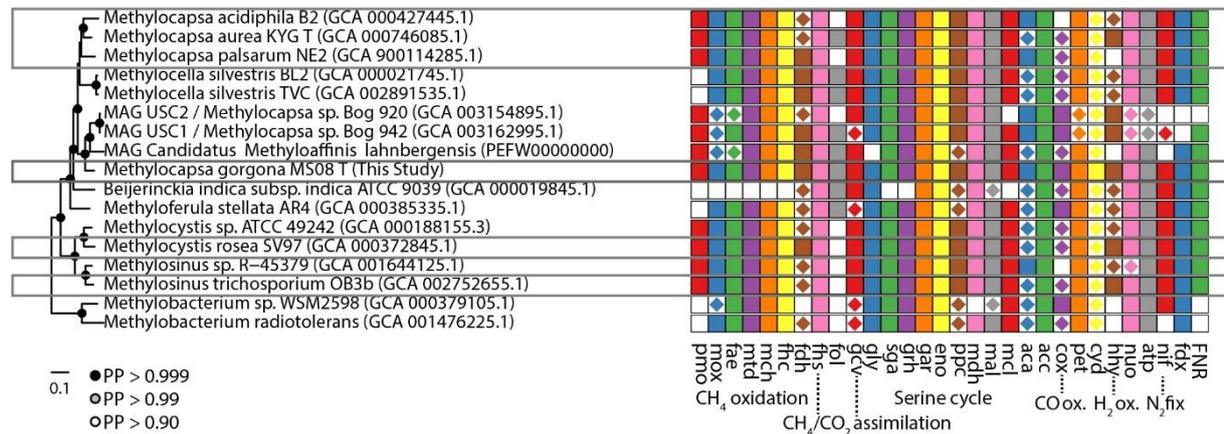
Figure 4. Phylogeny of the six respective methanotrophic strains M. acidiphila B2, M. aurea KYG, M. palsarum NE2, M. gorgona MG08, M. rosea SV97, and M. trichosporium OB3b based on 34 marker genes, and the presence/absence of functional complexes encoded in the genome of the respective strains. The methanotrophic strains are highlighted by the grey box. Presence of a complete complex is indicated by a solid square. Complexes that are incomplete are indicated by an embedded diamond. Abbreviations for functional complexes: aca, carbonic anhydrase; acc, acetyl-CoA carboxylase; atp, ATP synthase; cox, carbon monoxide dehydrogenase; cyd, terminal c cytochrome oxidase; eno, enolase; fae, formaldehyde activating enzyme; fdh, formate dehydrogenase; fdx, ferredoxin,2Fe-2S; fhc, formyltransferase/hydrolase complex; fhs, formate–tetrahydrofolateligase; FNR, ferredoxin-NADP+oxidoreductase; fol, bifunctional 5,10-methylene-tetrahydrofolatedehydrogenase, and 5,10-methylene-tetrahydrofolatecyclohydrolase; gck, 2-glycerate kinase; gcv, glycine cleavage complex; gly, serinehydroxymethyltransferase; hhy, [NiFe] hydrogenase; hpr, hydroxypyruvate reductase; mch, methenyl tetrahydromethanopterin cyclohydrolase; mcl, malyl-CoA lyase; mdh, malate dehydrogenase; mtd, NAD(P)-dependent methylene tetrahydromethanopterin dehydrogenase; mtk, malate thiokinase; mxa,methanol dehydrogenase; nif, nitrogenase; nuo, NADH-quinone oxidoreductase; pet, ubiquinol-cytochromecreductase; pmo, particulate methane mono-oxygenase; ppc, phosphoenolpyruvate carboxylase; and sga, serine–glyoxylate aminotransferase[101].

Methylocystis rosea SV97 is a non-motile, rod-shaped, 1.1–2.5 mm long and 0.8–1.1 mm wide, mesophilic methanotroph[106] (Fig. 5). It was isolated from a wetland soil showing minor CH_4 emissions near Ny-Ålesund, Svalbard, Norway. It grows at temperatures ranging from 5 to 37°C and a pH from 5.5 to 9.0. Like *M. trichosporium* OB3b, it accumulates PHB, fixes N_2 and encodes complete pMMO, nitrogenase, and Hhy complexes, and an incomplete CODH complex (Fig. 4).
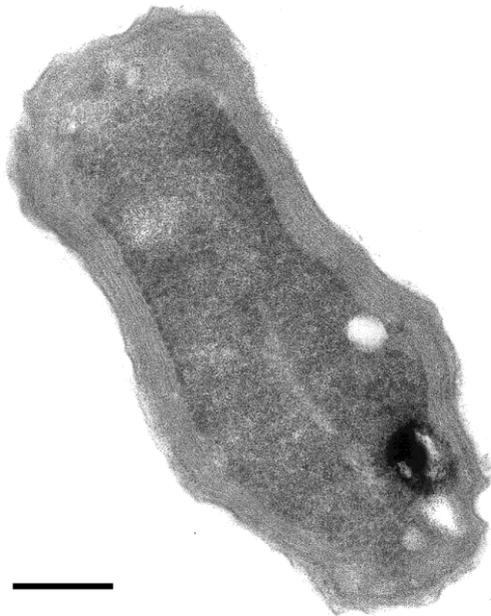
Figure 5. Transmission electron micrograph of a cell of *Methylocystis rosea* SV97 showing the type II intracytoplasmic membranes aligned with the periphery of the cell and inclusions (white spot). Bar, 200 nm.[106]

1.9.2 *Methylocapsa*

Methylocapsa acidiphila B2, *Methylocapsa aurea* KYG, *Methylocapsa palsarum* NE2, and *Methylocapsa gorgona* MG08 comprise, except the recently isolated *Methylocapsa polymorpha*[114,115], all the so far isolated species of the genus *Methylocapsa* and they all form colonies when incubated with air as the only carbon and energy source. The genus belongs to the family *Beijerinckiaceae* within the *Alphaproteobacteria*. Methanotrophic members of the family *Beijerinckiaceae* are gram-negative, moderately acidophilic, and use the serine pathway for carbon assimilation[19].

Methylocapsa acidiphila B2 is a non-motile, curved coccoid shaped strain. The cells are 0.5 – 0.8 µm wide and 0.8 – 1.2 µm long. It grows within a temperature range of 10 – 30 °C and a pH range of 4.2 – 7.2. It fixes N_2[107], accumulates PHB, possesses a full pMMO and nitrogenase complex, an incomplete Hhy complex and completely lacks the functional complex for CO oxidation[101] (Fig. 4).

Methylocapsa aurea KYG was isolated from a soil sample collected from under a small ephemeral brook in a forest near Marburg, Germany. The cells are non-motile, slightly curved rods, 0.7 – 1.2 µm wide and 1.8 – 3.1 µm long (Fig. 6). *M. aurea* KYG can fix N_2 under reduced

O$_2$ tension. It grows at temperatures ranging from 2 – 33 °C[108] and a pH of 5.2 and can accumulate PHB. Its genome encodes complete complexes for a pMMO, a nitrogenase, and a Hhy. The CODH complex is incomplete.
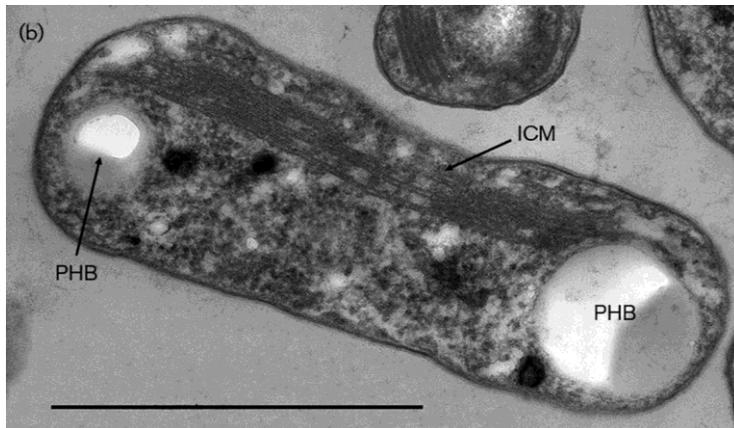
Figure 6. Electron micrograph of an ultrathin section of a methane-grown cell of strain KYG. ICM, Intracytoplasmic membrane; PHB, poly-β-hydroxybutyrate. Bar, 1 μm.[108]

Methylocapsa palsarum NE2 was isolated from a wet moss collected at a collapsed palsa site in northern Norway [109]. It is non-motile and forms slightly curved, 1.0 – 1.2 µm wide and 1.6 – 2.4 µm long rods (Fig. 7). It grows within a range of 6 to 32 °C and a pH range of 4.1 – 8.0. *M. palsarum* NE2 can fix N$_2$ under reduced O$_2$ tension. The strain encodes complete complexes of pMMO, and nitrogenase. The CODH complex is incomplete and it does not carry genes encoding for Hhy.
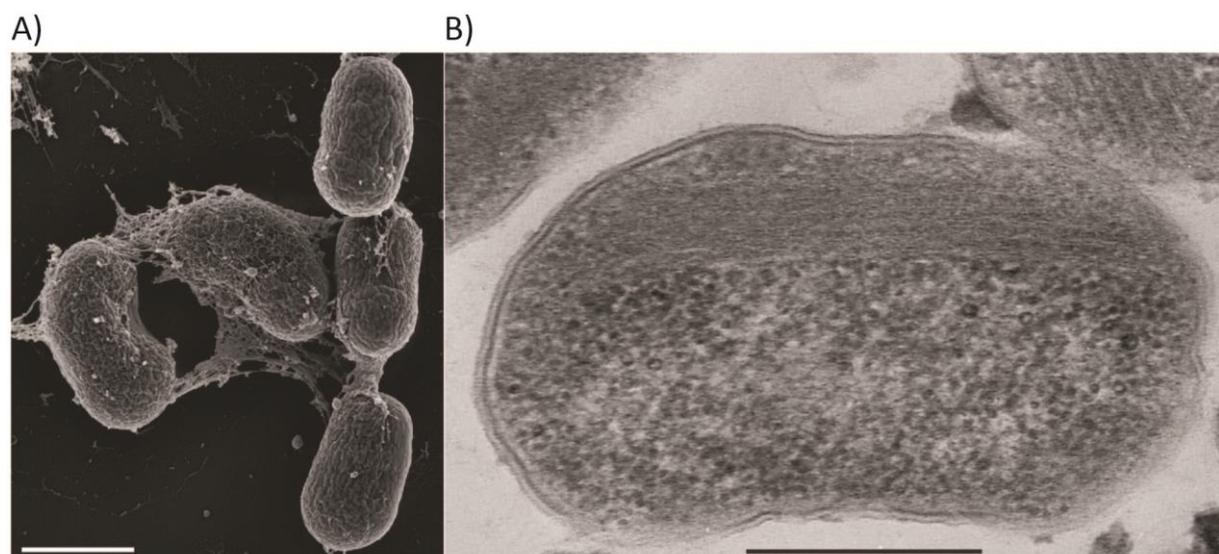
Figure 7. A) Scanning electron micrograph of *Methylocapsa palsarum* NE2 cells. B) Electron micrograph of an ultrathin section of *Methylocapsa palsarum* NE2 cells. Bars, A) 1 μm. B) 0.5 μm.[109]

Methylocapsa gorgona MG08 was isolated from cover soil sampled in a ditch at a retired subarctic landfill in northern Norway[101]. *M. gorgona* MG08 cells are non-motile, thick rods that are 0.6 – 0.8 µm wide and 0.8 – 1.5 µm long (Fig. 8). It grows within a range of 7 – 37 °C and a pH of 6 – 7.5. It can fix N_2 and encodes complete complexes for pMMO, nitrogenase, Hhy, and CODH.
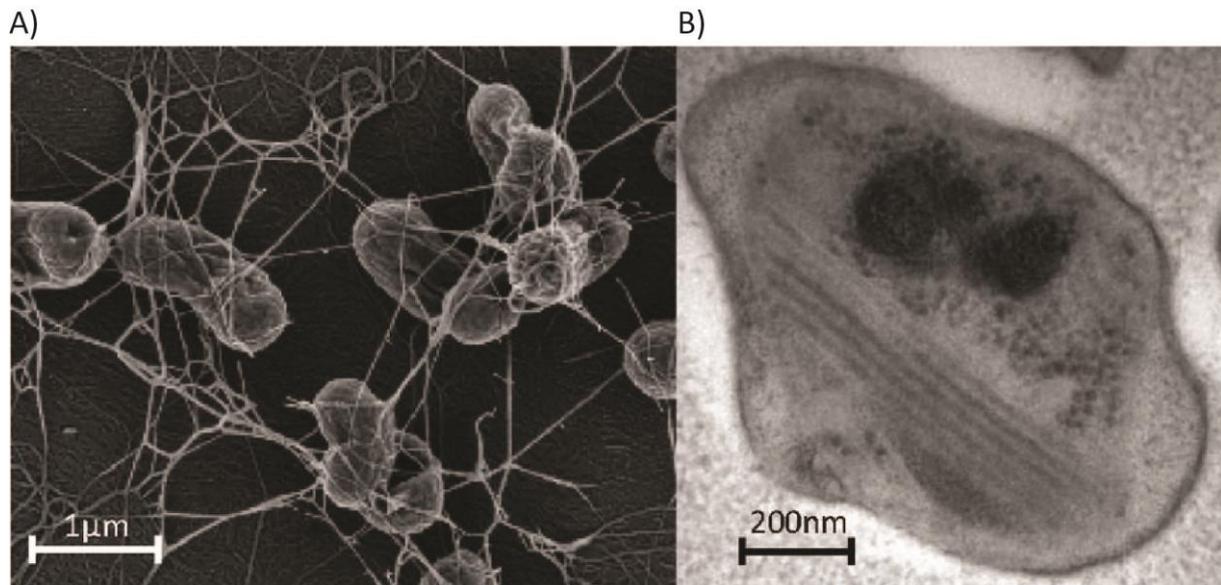
Figure 8. A) Scanning electron micrograph of *M. gorgona* MG08 cells. B) Transmission electron micrograph of a *M. gorgona* MG08 cell showing intracytoplasmic membranes and inclusions that resemble PHB granules. [101]

1.10   Aerobic methane oxidation

1.10.1 Activation of CH_4

Aerobic methanotrophs oxidize CH_4 to CO_2 using O_2 as electron acceptor[11]. The first step of the oxidation is the activation and the conversion of CH_4 to methanol by the methane monooxygenase (MMO). Two forms of MMOs are known. The soluble methane monooxygenase (sMMO) that occurs in the cytoplasm of methanotrophs and the particulate methane monooxygenase (pMMO) which is bound to intracellular membranes[116]. In contrast to the sMMO, the pMMO is found in almost all methanotrophs[117]. The sMMO, an enzyme consisting of a hydroxylase, a reductase, and a regulatory protein, employs a diiron cluster as catalytic site [118]. The pMMO, comprises the three subunits PmoA, PmoB, and PmoC that are organized in a trimer of PmoA, PmoB, and PmoC protomers, and requires copper for its activity[119,120]. As enzyme preparation techniques for structural analysis caused structural

changes and decreasing activity of the pMMO, the location and composition of its active site in still uncertain. Recent attempts to reconstitute the pMMO on nanodiscs to mimic the cellular environment of the pMMO restored its activity and revealed its structure in a lipid (membrane like) environment substantially revising the view of the pMMO[120].

For the oxidation of CH_4 to methanol (CH_3OH), the MMOs need CH_4, O_2, and a reductant (Fig. 9) to reduce the excess oxygen atom of O_2 to water:

$$CH_4 + O_2 + 2H^+ + 2e^- \rightarrow CH_3OH + H_2O$$
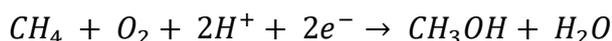
NADH/H^+ acts as the reductant for the sMMO. In case of the pMMO, hydroquinone derivates, NADH, methanol, succinate, and H_2 have been identified as electron donor *in vitro*[121–123], but how the electrons are transferred to the catalytic center of the pMMO is still unclear.

1.10.2 Methanol oxidation

The next step during the oxidation of CH_4 to CO_2 is the oxidation of the intermediate methanol to formaldehyde (CH_2O) (Fig. 9):

$$CH_3OH + PQQ \rightarrow CH_2O + PQQH_2$$
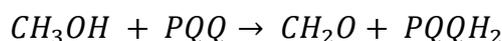
The reaction is catalyzed by a periplasmic, pyrroloquinoline quinone (PPQ) linked methanol dehydrogenase (MDH), a heterotetrameric enzyme consisting of two large and two small subunits[124]. The catalytic center can either contain a calcium (Ca^{2+}) or a lanthanide (Ln^{3+}) ion[125,126]. The MDH oxidizes methanol by reducing the prosthetic PQQ to the corresponding quinol and subsequently a two-step electron transfer to cytochrome c completes the oxidation. This oxidation step is accompanied by the generation of a proton gradient. The protons are retained in the cytoplasm while the electrons are transferred via cytochromes through a cytoplasmic membrane to a terminal oxidase. There, oxygen accepts the electrons and consumes protons from the cytoplasm. The electron transfer from MDH to oxygen is thought to operate several proton translocation segments[117]. Additionally, MDHs are known to oxidize methanol to formate ($HCOO^-$), but with a considerably lower conversion rate and substrate affinity[124].

1.10.3 Formaldehyde oxidation

The formaldehyde that results from the oxidation of methanol is transported to the cytoplasm (Fig. 9). There, it can be utilized in two different ways as described by Trotsenko and Murrell[117]. Either it is oxidized via formate to CO_2, or it can be assimilated via two different pathways, the Quayle ribulose monophosphate pathway (RuMP) or the serine cycle. As the methanotrophs, emphasized on in this work, apply the serine cycle for formaldehyde assimilation and tetrahydromethanopterin- (H_4MPT) and tetrahydrofolate- (H_4F) dependent routes for formaldehyde oxidation, only these pathways are described in the following. It is assumed that the H_4MPT-dependent pathway is the major catabolic route for formaldehyde oxidation[127], while the enzymes involved in the H_4F-depentent route mainly seem to maintain a high level of intermediates to feed the assimilatory serine cycle[128].

The first step of the H_4MPT-dependent formaldehyde oxidation is the condensation of formaldehyde and H_4MPT to methylene-H_4MPT:

$$CH_2O + H_4MPT \rightarrow CH_2 = H_4MPT + H_2O$$

This condensation either occurs spontaneously or can be catalyzed by a methylene-H_4MPT-specific formaldehyde-activating enzyme (Fae)[128–130].

The second step is the oxidation of methylene-H_4MPT to methenyl-H_4MPT:

$$CH_2O = H_4MPT + NAD(P)^+ \rightarrow CH_2 \equiv H_4MPT + NADPH$$
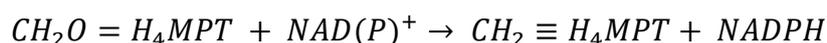
The methylene-H_4MPT can be oxidized by the methylene-H_4MPT dehydrogenase MtdA, that also catalyzes the reversible dehydrogenation of methylene-H_4F, or the methylene-H_4MPT-specific dehydrogenase MtdB[131,132]. The oxidation of methylene-H_4MPT to methenyl-H_4MPT by MtdA and MtdB is exergonic and thus, proceeds essentially irreversible. This plus the Fae activity supports an efficient and quantitative conversion of formaldehyde via the H_4MPT-dependent route[128].

Next, the methenyl-H_4MPT cyclohydrolase Mch hydrolyzes methenyl-H_4MPT to formyl-H_4MPT[133]:

$$CH_2 \equiv H_4MPT + H_2O \rightleftarrows CHO - H_4MPT$$
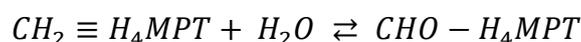
Then, the formyltransferase/hydrolase complex Fhc converts formyl-H_4MPT to formate (CHOO$^-$)[134]:

$$CHO - H_4MPT + H_2O \rightleftarrows CHOO^- + H_4MPT$$
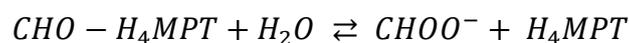
Finally, the resulting formate is oxidized to CO_2:

$$CHOO^- + NAD^+ \rightleftarrows CO_2 + NADH$$
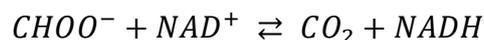
A NAD$^+$-dependent formate dehydrogenase (Fdh) is involved in this last step of the CH_4 oxidation (Fig. 9). Fdhs have been considered to catalyze the oxidation of formate to CO_2 nearly irreversible[135]. However, it has been shown that certain Fdhs reduce CO_2 to formate as well[136], and thus represent a potential entry point of CO_2 for carbon assimilation in non-phototrophic organisms.

The H_4F-dependent oxidation of formaldehyde to CO_2 (Fig. 9), that is thought to primarily feed the assimilatory serine cycle, operates in the oxidative or reductive direction depending on the requirements of the cell, as its reactions are fully reversible[117,128,137]. The condensation of the reactive formaldehyde and H_4F proceeds spontaneously:

$$CH_2O + H_4F \rightleftarrows CH_2 = H_4F + H_2O$$

The methylene-H_4F can either enter the glycine cleavage/synthesis (GCS) system that synthesizes glycine out of methylene-H_4F, NADH, NH_3, and CO_2[138], the serine cycle for further assimilation, or it can be oxidized. The oxidation is catalyzed by a MtdA or a bifunctional methylene-H_4F dehydrogenase/methylene-H_4F cyclohydrolase (FolD) to methenyl-H_4F:

$$CH_2O = H_4F + NAD(P)^+ \rightleftarrows CH_2 \equiv H_4F + NADPH$$
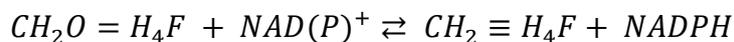
During the next step of the oxidation, the resulting methenyl-H_4F is hydrolyzed to formyl-H_4F by a FolD or a methenyl-H_4F cyclohydrolase (Fch):

$$CH_2 \equiv H_4F + H_2O \rightleftarrows CHO - H_4F$$
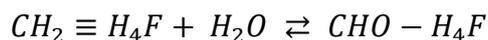
The formyl-H_4F is then oxidized to formate by a formate-tetrahydrofolate ligase (Fhs). The reaction is coupled to the formation of adenosine triphosphate (ATP)[139]:

$$CHO - H_4F + ADP + P_i \rightleftarrows CHOO^- + H_4F + ATP$$
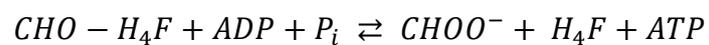
In general, the dehydrogenases that take part in the oxidation from CH_4 to CO_2 are coupled to the energy conservation, while the oxidases are usually not energy conserving[140]. In total, CH_4 oxidation yields six electrons usable for aerobic respiration, carbon fixation, or the activation of CH_4[119].
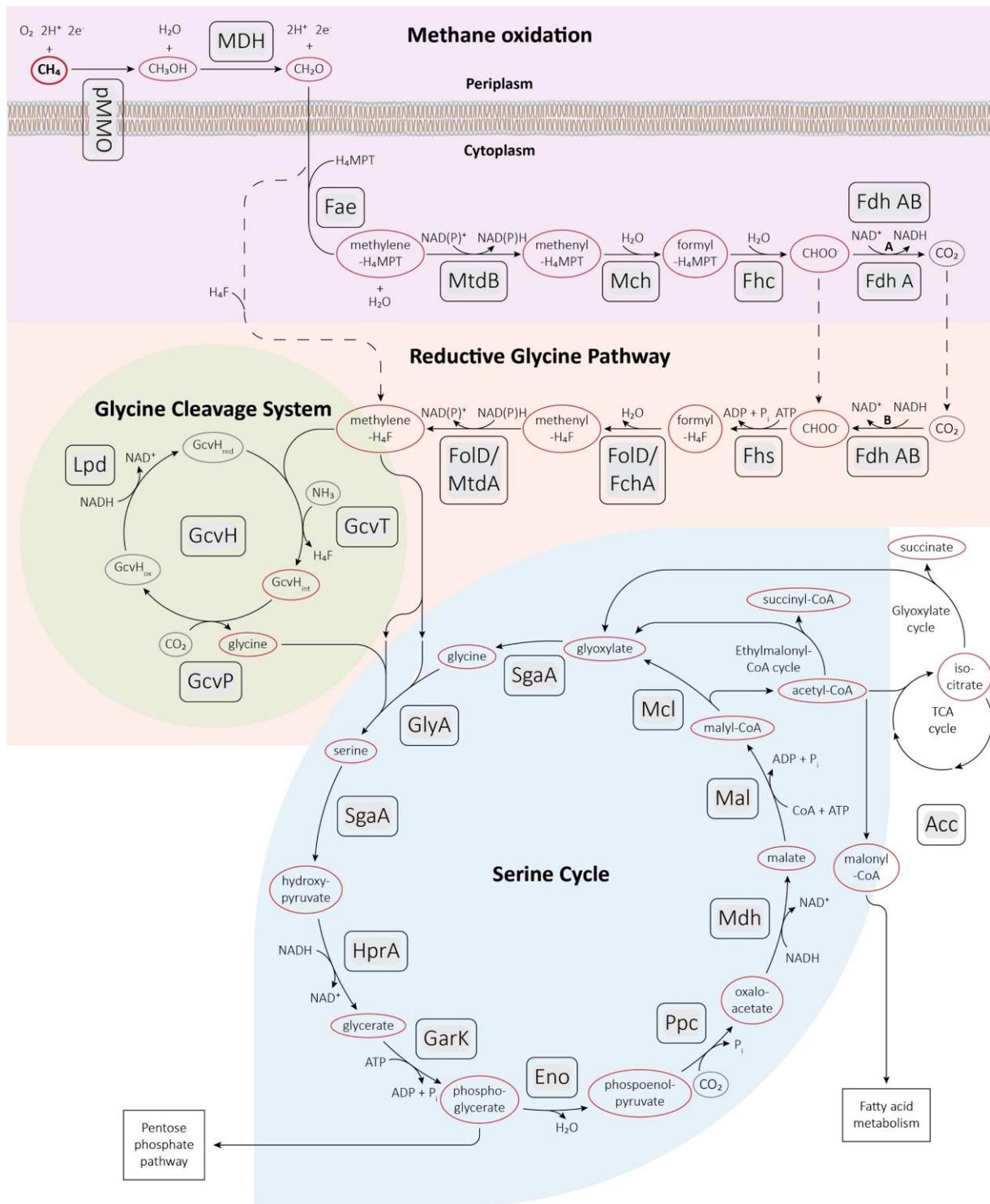
Figure 9. pMMO – particulate methane monooxygenase, MDH – methanol dehydrogenase (Mox) and putative lanthanide-dependent methanol dehydrogenase (XoxF), Hhy – [NiFe] group 1h hydrogenase, CODH – [MuCO] class I carbon monoxide dehydrogenase, Fae – formaldehyde activating enzyme, MtdB – NAD(P)-dependent methylene-tetrahydromethanopterin dehydrogenase, Mch – methenyl-tetrahydromethanopterin cyclohydrolase, Fhc – formyltransferase/hydrolase, Fdh **A** – NAD-dependent formate-dehydrogenase, Fdh **AB** – molybdopterin binding reversible formate dehydrogenase/CO₂ reductase, Fhs – formate-tetrahydrofolate ligase, FolD – bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/ 5,10-methenyl-tetrahydrofolate cyclohydrolase, FchA – methenyltetrahydrofolate cyclohydrolase, MtdA – NADP-dependent methylenetetrahydrofolate dehydrogenase, GcvH – glycine cleavage system H protein, GcvT – aminomethyltransferase, GcvP - glycine dehydrogenase, Lpd

33

– dihydrolipoyl dehydrogenase, GlyA – serine hydroxymethyltransferase, SgaA – Serine-glyoxylate aminotransferase, HprA – glycerate dehydrogenase, GarK – 2-glycerate kinase, Eno – enolase, Ppc – phosphoenolpyruvate carboxylase, Mdh – malate dehydrogenase, Mal – malate-CoA ligase, Mcl – L-malyl-CoA lyase, Acc – Acetyl-CoA carboxylase, NAD^+ – nicotinamide adenine dinucleotide, $NADP^+$ – nicotinamide adenine dinucleotide phosphate, ATP – adenosine triphosphate.

1.10.4 Assimilation of carbon via the glycine cleavage system and the serine cycle

As mentioned above, the formaldehyde that results from the oxidation of CH_4 and methanol can spontaneously condense with H_4F to methylene-H_4F and then enter the glycine cleavage system (GCS) or the serine cycle (Fig. 9). Alternatively, the formaldehyde is oxidized via the H_4MPT-mediated route to formate. The formate is then reduced via the H_4F-mediated route to methylene-H_4F before entering the GCS or the serine cycle. Studies involving *Methylorubrum extorquens* AM1 have demonstrated that mutants defective in any of the three enzymes involved in the H_4F mediated formaldehyde oxidation could not grow on methanol implying that this pathway plays an important role in carbon assimilation[139,141]. Later, it was shown that the reductive pathway via formate to methylene-H_4F and not the spontaneous condensation of formaldehyde and H_4F represents the major assimilatory flux[142]. Another possibility to assimilate carbon is the reduction of CO_2 to formate by a reversible Fdh and the further reduction to methylene-H_4F[143].

The GCS consists of four loosely associated enzymes and converts the methylene-H_4F, NH_3, CO_2, and NADH, to glycine, H_4F and NAD^+:

$$CH_2O = H_4F + NH_3 + CO_2 + NADH \rightleftarrows Glycine + H_4F + NAD^+$$

The conversion of formate and CO_2 to the C_2-amio acid glycine is referred to as the core module of the reductive glycine pathway[144] (Fig. 9).

Next in carbon assimilation is the serine cycle (Fig. 9). As summarized by Anthony[145,146], the serine cycle differs from other formaldehyde assimilation pathways as its intermediates are carboxylic acids and amino acids instead of carbohydrates. It drives the conversion of the C_1 compound formaldehyde to C_3 and C_4 intermediates and thereby provides precursors for further biosynthesis. Two molecules of formaldehyde and two of glycine result in two molecules of phosphoglycerate. One phosphoglycerate is assimilated while the other is carboxylated to phosphoenolpyruvate and further converted via oxaloacetate to malyl-CoA. The malyl-CoA is

cleaved to glyoxylate and acetyl-CoA. The glyoxylate is converted to glycine, while there are two different variants of the serine cycle to oxidize the acetyl-CoA to glyoxylate[145,146]. One variant involves the glyoxylate cycle (encoded by the *Methylocapsa* strains) to oxidize acetyl-CoA to glyoxylate, the other variant involves the ethylmalonyl-CoA cycle for the oxidation (encoded by the *Methylocystis rosea* SV97). The energy and carbon balances of both variants normalized to the production of oxaloacetate (OAA)[146] are:

$$2CH_2O = H_4F + 2CO_2 + 2NAD(P)H + FAD + 3ATP$$
$$\rightleftarrows OAA + 2H_4F + 2NAD(P)^+ + FADH_2 + 2ADP + 2P_i + AMP + PP_i$$
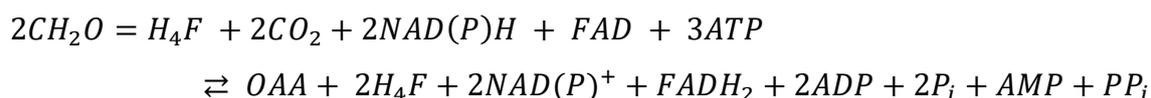
for the glyoxylate cycle based serine cycle, and:

$$3CH_2O = H_4F + 5CO_2 + 6NAD(P)H + FAD + 5ATP$$
$$\rightleftarrows 2OAA + 3H_4F + 6NAD(P)^+ + 2FADH_2 + 3ADP + 3P_i + 2AMP + 2PP_i$$
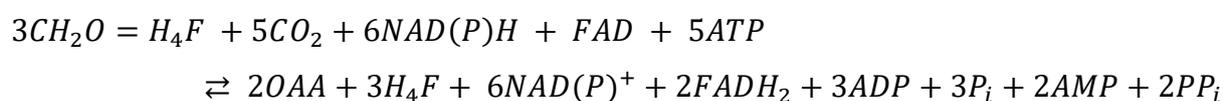
for the ethylmalonyl-CoA cycle.

1.11 Oxidation of atmospheric hydrogen and carbon monoxide

Besides CH_4, the most abundant atmospheric trace gases that represent potential energy sources for microorganisms are H_2 and CO with average atmospheric concentrations of 530, and 90 ppb[81,119], respectively. As reviewed by Greening and Grinter[93], hydrogenotrophic and carboxydotrophic microorganisms, that oxidize atmospheric concentrations of H_2 and CO, respectively, are assumed to grow heterotrophically while they oxidize H_2 and CO to cover their cellular maintenance energy to persist periods of starvation enabling long-term survival[147–149]. To do so, these microorganisms have evolved enzymes with high affinities that are reflected in $K_{m(app)}$ values smaller than 150 nM for the respective gases[119]. The high affinity oxidation of H_2 and CO is attributed to [NiFe] hydrogenases within different and distantly related groups, named Hhy, Huc, Hyo, and Hyl and molybdenum-dependent carbon monoxide dehydrogenases (CODH), respectively[119]. Both enzymes are metalloenzymes consisting of multiple subunits. All known high affinity hydrogenases comprise two subunits, a small and a large one. In Hhy, the hydrogenase encoded in some of the six investigated methanotrophic strains, the two subunits are arranged in a protomer. Two of these protomers dimerize to the final hydrogenase[150]. The high affinity CODH consist of three subunits, a small, a medium, and a

large subunit organized in a trimer. Two trimers dimerize to form the enzyme[119,150,151]. Both of high affinity enzymes seem to be anchored at the cytolytic side of the inner membrane[152–154]. Until recently, the measurements describing the high affinity oxidation of H_2 and CO were solely based on whole cell kinetics. However, in 2023, a Huc hydrogenase (K_m = 129 nM) was successfully isolated, and its kinetics characterized[155]. The electrons from the oxidation of H_2 are thought or, in case of the isolated hydrogenase, shown to be directly transferred into the respiratory chain[119,155]:

$$H_2 \; \rightarrow \; 2H^+ + 2\,e^-$$

The same applies to the electrons from the oxidation of CO with H_2O to CO_2:

$$CO + H_2O \; \rightarrow \; CO_2 + 2H^+ + 2\,e^-$$

1.12 N_2-fixation by methanotrophs

The ability of MOB to fix N_2 has been confirmed for representatives within *Alpha-* and *Gammaproteobacteria*, and *Verrucomicrobiota*[156,157]. Complete structural genes (*nifHDK*) for the molybdenum-dependent isozyme of the nitrogenase, the enzyme that catalyzes the biological reduction of N_2 to ammonia (NH_3), have been found in 98% of the methanotrophs within the *Alphaproteobacteria* and in 89% within the *Gammaproteobacteria*, suggesting that MOB play an important role in N_2-fixation[158–160]. As methanotrophs require approximately 0.25 mole nitrogen per mole carbon assimilated, their nitrogen requirements are relatively high[69,145]. Thus, the ability to fix N_2 seems to be a necessity to prevent cellular nitrogen limitations in oligotrophic environments. To fix N_2, MOB need to overcome two major obstacles, the irreversible inactivation of the nitrogenase by oxygen and the high energy requirements of the fixation of N_2[161]. Especially during the oxidation of atmospheric CH_4 by atmMOB, these obstacles seem to be problematic as atmMOB are exposed to high O_2 tensions while facing an apparent energy limitation. In free-living diazotrophs (N_2-fixing microorganisms), heterocysts, aggregation of individual bacteria, and a high respiratory activity can create a nearly anoxic environment to protect the nitrogenase from inactivation by O_2[162–164]. Besides, a conformational protection, during which the nitrogenase forms a reversible state that is oxygen-tolerant but inactive, enables survival of nitrogenases during oxygen stress[165]. The high energy

requirements to break the N-N triple bond of N_2 that represents one of the strongest bonds in nature and the further reduction to ammonia is reflected by the optimal stoichiometry of the reaction[160,166,167]:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$
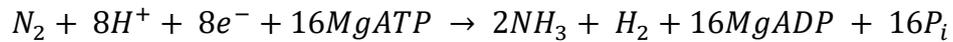
All six strains investigated within this thesis encode a molybdenum-iron nitrogenase (Nif)[101,168]. This nitrogenase is a two component metalloprotein that consists of an iron (Fe) and a molybdenum iron (MoFe) protein. The Fe protein is a homodimeric ATPase and the obligate reductase of the nitrogenase that couples ATP hydrolysis to the electron transfer to the MoFe protein[160,166]. The MoFe protein is a α2β2 tetramer with two metalloclusters and a MoFe cofactor being the active site where N_2 is bound and reduced[166]. During N_2 fixation the MoFe protein undergoes various reduced states that are required to bind and reduce N_2. The binding of N_2 and the reduction of the triple bond follows a reductive elimination reaction that is associated with the evolution of H_2[169].

2 Material and Methods

2.1 Filter cultivation

Key to grow atmMOB with air as sole carbon and energy source (hereafter referred to as growth "on air") was the cultivation of cells on polycarbonate filters that float on a defined, carbon-free medium (hereafter "filter cultivation") (Fig. 10, 11, 12.1). This method is based on filter cultivation techniques that were originally invented to isolate "uncultivable" microorganisms by simulating natural environments[170,171]. A major advantage of filter cultivation over conventional cultivation techniques using liquid media is the superior mass transfer of gases to the cells, as the cells are only surrounded by a thin boundary layer of medium (Fig. 10). This enables diffusion of atmospheric trace gases to the cells quick enough to support growth, while media is supplied. Further, the cells that are immobilized on the supporting filter cannot escaped detection. Thus, growth of cells leading to colony formation can be observed using microscopy (Fig. 10).
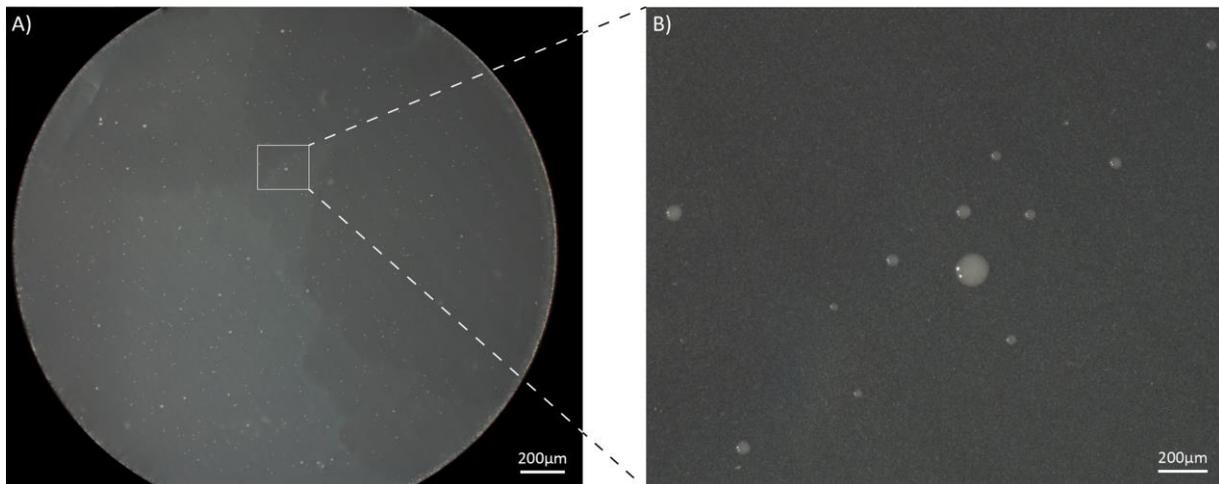
Figure 10. A) Light microscopic image of a *Methylocapsa gorgona* MG08 culture on a polycarbonate filter after a 12-month incubation period on a carbon- and nitrogen-free growth medium. B) Magnified image section of the filter colony shown in A). Single colonies of cells coated by thin boundary layer.

To create filter cultures, the methanotrophic strains were cultivated in a serum bottle using liquid medium and high CH_4 concentrations and used as inoculum (Fig. 12.1). A fraction of this inoculum was filtered on polycarbonate filter supported by a glass fiber filter using a filtration manifold (Fig. 12.1) and applying vacuum. During this step, the inoculum was rinsed in the manifold cylinder with a suitable washing solution (e.g., growth medium). After the filtration, the thinly populated filter (Fig. 12.1) was moved on growth medium in a petri dish. The petri

dish enabled aeration and by that constant substrate supply while keeping the filter culture sterile. Because of the slow growth of atmMOB on air, filter cultures needed to be incubated for several months to form colonies big enough to confirm growth and to accumulate sufficient biomass for further experiments (Fig. 12).

2.2 Oxidation of atmospheric trace gases and energy yield estimation

The oxidation of atmospheric CH_4, H_2, and CO and the estimation of the energy yield from the oxidation of these gases were assessed by conducting oxidation experiments. Therefore, filter cultures were moved to glass bottles containing a defined volume of growth medium. The bottles were sealed airtight with suitable caps (Fig. 12.2). By purging the headspace of the glass bottles with synthetic air containing 400 ppm CO_2 and adding CH_4, H_2, and CO manually using a gas syringe, a defined headspace was created (Fig. 12.2). To assess the oxidation rates, the filter cultures were incubated at a constant temperature and the change of the CH_4, H_2, and CO in the headspace over time was measured via gas chromatography. For each measurement, the gas from the headspace was sampled using a gas tight syringe. In addition, the pressure in the headspace was measured using a manometer. By knowing the gas volume and the gas pressure in the bottle headspace, the temperature during incubation, and the relative concentration of the respective gas species, the mass of each trace gas at the different sampling timepoints was calculated via the ideal gas law. As the low trace gas concentrations were rate-limiting (reflected by an oxidation rate proportional to the concentration of the trace gas), pseudo first order kinetics for the oxidation of atmospheric CH_4, H_2, and CO were assumed. By knowing the change in mass of the gases over time, the reaction rate constant could be calculated using the integrated rate law for first order reactions. By knowing the reaction rate constant, the oxidation rates of the respective gases by the filter cultures at atmospheric concentrations could be calculated. To determine the oxidation rates per cell, the number of cells within the filter cultures was quantified (see section 2.2.1). Under consideration of the Gibbs free energy changes for the oxidation of the respective gases at atmospheric concentrations and the cellular oxidation rates, the energy yield per cell was calculated. To compare the energy yield from substrate consumption and thus the energy sufficient for maintenance and growth of different microbial species, the energy yield per cell was normalized to dry mass carbon and hour (kJ C-

mol^{-1} h^{-1}). This was achieved by considering the carbon content (see section 2.2.2), the cellular dry weight, and the cellular energy yield during trace gas oxidation of the respective species.

2.2.1 Quantification of cells in filter cultures

The quantification of the cell numbers in filter cultures is challenging due to the formation of multilayered colonies during growth (Fig. 11). This prevents direct quantification of cell numbers on the filter via microscopy. As consequence, two different approaches were employed to quantify cell numbers of filter cultures for the publications included in this thesis.
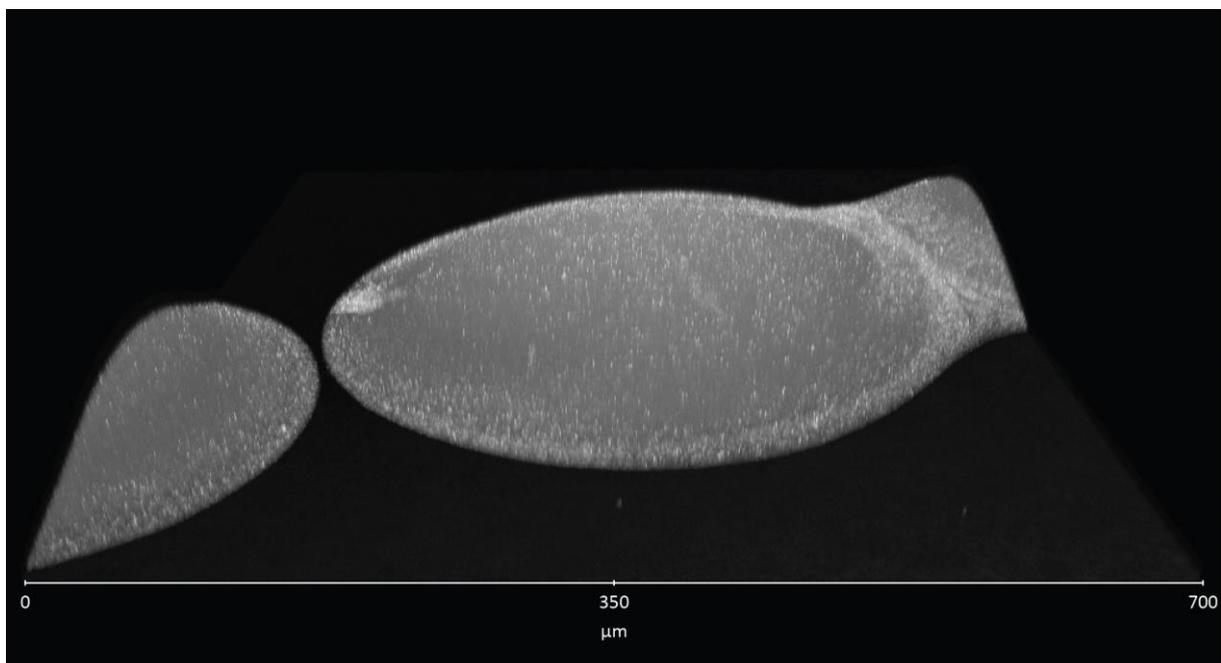
Figure 11. Stacked confocal images of SYBR green II stained *Methylocapsa palsarum* NE2 cells in colonies after long-term incubation on air.

For paper 1, the cell numbers were determined by comparing the DNA mass of filter cultures to the DNA masses of known cell numbers from liquid cultures of the same species. For paper 2, cells on the filters were stained using a fluorescent nucleic acid stain, washed from the filters, and collected in tubes. The collected and stained cells were counted using flow cytometry (Fig. 12.2).

2.2.2   Cellular dry mass estimation and carbon content

As for the cell quantification, the cellular dry mass of the methanotrophic strains was determined differently for both papers. For paper 1, the cell concentration of a liquid culture was determined via microscopy. A defined amount of this liquid culture with known cell concentration was pelleted in a tube via centrifugation and the supernatant discarded. The resulting pellet was dried in an oven and weighed. The cellular dry weight was calculated by dividing the weight of the dry pellet by the number of cells pelleted. For paper 2, the cellular dry weight was determined by measuring single-cell buoyant mass distributions of cells from filter cultures in H_2O-based and deuterium oxide (D_2O)-based solutions of phosphate saline buffer (PBS) using a suspended microchannel resonator (SMR)[172–174]. The SMR is basically a "balance" that consist of a vibrating microcantilever with an internal microfluidic channel. The SMR oscillates at a frequency proportional to its mass. When a cell, that is transported by a solution of known density, passes through the channel, the resonance frequency of the cantilever changes by an amount proportional to the cells buoyant mass[173]. To determine the cellular dry mass, buoyant mass measurements in two fluids of differing densities (H_2O-based and D_2O-based solutions of PBS) were required[174].

To determine the cellular carbon content, the methanotrophic strains were grown in stirred-tank bioreactors to receive sufficient biomass. The harvested biomass was washed to remove the growth medium and lyophilized. The cellular carbon content of the methanotrophic strains was determined using an elemental analyzer coupled to an isotope ratio mass-spectrometer.

2.3 N_2 fixation by atmMOB

To evaluate if the methanotrophic strains can fix N_2 during growth on air, filter cultures were incubated with air as sole carbon, energy, and nitrogen source. After accumulation of sufficient biomass, the filter cultures were incubated in sealed bottles with air and trace concentration of CH_4, H_2, and CO as mentioned (see section 2.2). Additionally, a known amount of $^{15}N_2$ was added to the bottle headspace. The rationale is that growing cells capable of fixing N_2, enrich ^{15}N isotopes in their biomass. These enriched ^{15}N isotopes can be detected using mass spectrometry. As growth on air by atmMOB is very slow, long incubation times for cellular ^{15}N enrichment were needed. Therefore, filter cultures of atmMOB were kept under a $^{15}N_2$

headspace for two months. After, cells were harvested by washing from filters and lyophilized. The relative ^{15}N to ^{14}N ratio of the cellular biomass was detected using Nanoscale secondary ion mass spectrometry (NanoSIMS) (Fig. 12.3). During NanoSIMS an primary cesium ion beam erodes the surface of a sample releasing atoms and molecular fragments. A fraction of these secondary particles is ionized. The secondary ions are characteristics of the sample region eroded and can be manipulated with ion optics. Therefore, the secondary ions can be directed to a mass spectrometer where the ions are measured[175]. Based on that, a quantitative atomic mass image of the analyzed sample region can be created. The NanoSIMS couples microscopy and isotopic analysis at high mass resolution and spatial resolution down to 50nm[176]. This enabled the detection of low ^{15}N enrichments in single atmMOB cells while only little biomass was required for analysis.

2.4   AtmMOB adjustments to growth on air

To assess the metabolic adjustments of atmMOB during growth on air, comparative proteomics experiments were performed. As proteins carry out a majority of cellular functions and are closely related to the phenotype of cells, comparative proteomics is a universal approach to unravel the differences between two cellular states with respect to the molecular mechanisms involved in various processes[177]. During these experiments, the proteome of an atmMOB strain incubated at elevated CH_4 concentrations was compared to the proteome of the same strain at atmospheric CH_4 concentrations (Fig. 12.4). The comparison of these two physiological states enabled to investigate the allocation of cellular investments into biological processes relevant for growth on air and thus, to some extent, how the atmMOB species manage to grow on air. Filter cultures grown with air as sole carbon and energy source grow very slowly and accumulate only little amounts of biomass. Thus, to minimize loss of biomass, the filter cultures were washed from the filter, collected (Fig. 12.2), and concentrated by lyophilization. For the identification and the relative quantification of proteins, proteins were extracted from the biomass and then digested to peptides using the enzyme trypsin. The resulting peptides were separated via liquid chromatography and analyzed using tandem mass spectrometry. The resulting peptide spectra were compared to a strain specific protein library. If a peptide spectrum matched the spectrum of a specific amino acid sequence predicted from the protein library, the match was counted as peptide spectrum match and the peptide was assigned to the

matching protein. Based on this approach for identification and quantification, the protein abundances of the two different treatments could be compared enabling the identification of major protein allocation, and thus metabolic adjustments, during growth on air.

1. Filter cultivation of atmMOB

① Cultivation at high CH₄ concentrations

Diluted inoculum

Polycarbonate filter
Glass fiber filter

① Filtration of cells on polycarbonate filters

Petri dish
Filter
Medium

① Incubation of filter cultures floating on medium

SYBR green stained cells after filtration

10 µm

SYBR green stained cell after incubation

10 µm

Biomass
Incubation of filter cultures with air as only energy and carbon source for several months

Energy yield during growth on air?

N₂ fixation on air?

20% ¹⁵N₂ in air

Incubation of filter cultures with air and ¹⁵N₂
③

3. ¹⁵N₂ in-corporation

Adjustments to growth on air?

Incubation of filter cultures at different CH₄ concentrations
④

1000 ppm CH₄   vs   1.9 ppm CH₄

4. Comparative proteomics

1000 ppm CH₄   1.9 ppm CH₄

Intensity

m/z   m/z

Extraction, digestion, and identification of proteins
④

Harvesting of cells
④
Vortex
③

Cs⁺   ¹²C¹⁴N⁻
        ¹²C¹⁵N⁻

Detection of cellular ¹⁵N using NanoSIMS
③

HPLC cap
Headspace
Medium
Filter
②

Synthetic air

H₂, CH₄, CO
+

2. Trace gas oxidation experiment

Creation of defined atmosphere
②

Incubation and GC measurements
②

Vortex

Stained cells

Staining and washing of filter cultures
②

Flow cytometer

SSC
FSC

Quantification of cells
②

44

Figure 12. Simplified overview of the most important methods and workflows used and established during the PhD to answer the research questions investigated. ① Basic cultivation steps to create filter cultures with sufficient biomass for further analysis. ② Simplified steps performed for the estimation of energy yields from the oxidation of the three trace gases CH_4, CO, and H_2 in air. ③ Simplified steps performed to test N_2 fixation of atmMOB exposed to air. ④ Simplified steps performed to determine the adjustments by atmMOB to growth on air via comparative proteomics.

2.5   Global distribution of atmMOB

To outline their global distribution, the environments atmMOB are found in, and eventually to gain a first impression of the ecological relevance of atmMOB, the publicly available 16S rRNA gene fragment collection of the Earth Microbiome Project (EMP) was screened for the atmMOB species investigated in this thesis as previously described[94]. The EMP is a large-scale collaborative project employing standardized collection, curation, and analysis of environmental samples[178–180]. Sample processing, sequencing, and core amplicon data analysis were performed by the EMP (www.earthmicrobiome.org), and all amplicon sequence data and metadata have been made public through the EMP data portal (qiita.microbio.me/emp). To obtain putative atmMOB 16S rRNA gene fragments, all EMP 16S rRNA gene fragments with >98.5% sequence identity to the 16S rRNA genes of *M. gorgona*, *M. aurea*, *M. palsarum, and M. rosea* were selected via BLASTN search[181]. In total, all the 23, 813 EMP samples from the three EMP datasets (emp_deblur_90bp.qc_filtered.biom, emp_deblur_100bp.qc_filtered, emp_deblur_150bp.qc_filtered.biom) that include sample location coordinates, representing 1294 unique geographic locations, were screened for the presence and abundance of the four atmMOB. R[182] (version 4.1.3) was used to analyze the environmental distribution and relative abundances of *M. gorgona*, *M. aurea*, *M. palsarum, and M. rosea* like 16S rRNA gene fragments and to graphically display the results (Rstudio version 2022.02.2+485). This was done using the R packages tidyverse version 1.3.1 (https://cran.r-project.org/web/packages/tidyverse/), maps version 3.4.0 (https://cran.r-project.org/web/packages/maps/), and mapdata version 2.3.0 (https://cran.r-project.org/web/packages/mapdata/).

3   Results

3.1   Paper 1: Simultaneous Oxidation of Atmospheric Methane, Carbon Monoxide, and Hydrogen for Bacterial Growth

This study builds upon the previously reported findings that *M. gorgona* MG08, the first member of the USCα in pure culture, can grow on air by oxidizing atmospheric CH_4 concentrations. In addition to a pMMO, *M. gorgona* MG08 encodes a [NiFe] group 1 high affinity respiratory hydrogenase (Hhy) and a [MoCu] class I respiratory carbon monoxide dehydrogenase[101]. Based on this, the capability of *M. gorgona* MG08 filter cultures to oxidize the trace gases H_2 and CO at their atmospheric concentrations, in addition to atmospheric CH_4, was tested. The major finding of the study was the simultaneous oxidation of atmospheric CH_4, H_2, and CO to sub-atmospheric concentrations by *M. gorgona* MG08 during growth on air. This demonstrated that a mixotrophic lifestyle, based on the three most abundant trace gases in the atmosphere that represent energy sources, enables *M. gorgona* MG08 to grow on air. Furthermore, it was shown that the oxidation of atmospheric CH_4 does not depend on the oxidation of H_2 and CO, while the oxidation of H_2 and CO proceed without the presence of CH_4. Based on the trace gas oxidation rates measured for *M. gorgona* MG08, the study reports the first energy yield estimates for an atmMOB pure culture during growth on air, namely 0.38 kJ C-mol^{-1} h^{-1}. This value, that represents an estimate for growth, is approximately 7.5 times lower than the basic premise assumed necessary for cellular maintenance stated in literature (2.8 kJ C-mol^{-1} h^{-1} at 20°C)[80]. However, the butyl rubber stoppers, used to seal the incubation bottles, emitted trace amounts of CO, and caused minor trace gases diffusion during the oxidation experiments. This might have impacted the accuracy of the estimations. Nevertheless, based on the study's findings, it was concluded that a metabolic flexibility enabling the mixotrophic oxidation of atmospheric CH_4, H_2, and CO and low energy requirements allow *M. gorgona* MG08 to grow on air.

3.2 Paper 2: Physiological Basis for Atmospheric Methane Oxidation and Methanotrophic Growth on Air

This study is based on two major questions that have remained partially unanswered since the discovery of atmospheric CH_4 oxidation by methanotrophs: Which methanotrophs are responsible for the oxidation of atmospheric CH_4? How can these organisms survive and grow despite the apparent energetic limitations inherent to the oxidation of the low atmospheric CH_4 concentrations? Besides the oxidation of atmospheric CH_4 by methanotrophs assigned to the proteobacterial USCα and USCγ, the presence of "conventional" methanotrophs in soils being net sinks for atmospheric CH_4 indicated that members of the genera *Methylocapsa*, *Methylosinus,* and *Methylocystis* might also contribute to atmospheric CH_4 oxidation. Therefore, the alphaproteobacterial, methanotrophic strains *Methylocystis rosea* SV97, *Methylosinus trichosporium* OB3b, *Methylocapsa acidiphila* B2, *Methylocapsa aurea* KYG, *Methylocapsa palsarum* NE2, in addition to *M. gorgona* MG08, and the gammaproteobacterial, methanotrophic strains *Methylobacter tundripaludum* SV96 were screened for their ability to grow with air as sole energy and carbon source. Further, by estimating the energy yield of atmMOB during growth on air via oxidation experiments, using comparative proteomics to investigate proteome allocations, and determining the a_A^0 for CH_4 during growth on air, the physiological basis of atmMOB was outlined. As the effect of nitrogen limitation on atmMOB has never been investigated, the ability of atmMOB to grow on nitrogen sources available in air was evaluated.

3.2.1 Colony formation, trace gas oxidation, and cellular energy yield during growth on air

Filter cultures of *M. rosea* SV97, *M. trichosporium* OB3b, *M. acidiphila* B2, *M. aurea* KYG, *M. palsarum* NE2, and *M. gorgona* MG08 formed colonies during incubation with air as sole energy and carbon source, while no colonies were formed by *M. tundripaludum* SV96 (thus, M. tundripaludum was not further investigated). Additionally, filter cultures of *M. gorgona* MG08 exposed to synthetic air without trace gases did not form colonies. During trace gas oxidation experiments, filter cultures of *M. rosea* SV97, *M. aurea* KYG, *M. palsarum* NE2, and *M. gorgona* MG08, incubated on air for six- and 12-months, enduringly oxidized atmospheric concentration of CH_4, H_2 and/or CO with strain specific oxidation patterns, while

filter cultures of *M. trichosporium* OB3b and *M. acidiphila* B2 were inactive. The absence of colony formation by *M. gorgona* MG08 in synthetic, trace gas free air and the enduring trace gas oxidation by *M. rosea* SV97, *M. aurea* KYG, *M. palsarum* NE2, and *M. gorgona* MG08 confirmed that these strains can live and grow with air as the sole energy and carbon source. Furthermore, the oxidation experiments revealed mixotrophy by all four strains, showed substantial metabolic differences between the strains, and demonstrated that the enduring oxidation of atmospheric CH_4 is not restricted to members of the USCα and USCγ.

The energy yield estimates from the oxidation of trace gases in air ranged from 0.38 to 0.71 kJ C-mol^{-1} h^{-1}, values 3.9 – 7.4 times lower than the reported average energy requirements for cellular maintenance in aerobic bacteria (2.8 kJ C-mol^{-1} h^{-1} at 20°C). This contradicted and revised the basic maintenance energy premise for atmMOB.

3.2.2 Comparative proteomics

To investigate the metabolic adjustments of *M. gorgona* MG08, *M. rosea* SV97, and *M. palsarum* NE2 during growth on air, the proteome allocation by filter cultures of the respective strains exposed to atmospheric CH_4 were compared to the proteome allocation to filter cultures exposed to 1000 ppm CH_4. This revealed major proteome shifts related to their core metabolism including transcription, translation, growth, energy metabolism, and amino acid and carbohydrate transport as adjustments during growth on air. In depth analysis of the abundance of proteins involved in trace gas oxidation demonstrated distinct metabolic strategies to oxidize trace gases. While *M. palsarum* NE2 allocated protein investments to CH_4 oxidation, *M. gorgona* MG08 and *M. rosea* SV97 increased investments in H_2 and CO oxidation during growth on air. This implied that the strains allocated resources to increase their a_A^0 for the respective trace gases as adjustments to grow on air as well as niche differentiation. Additionally, investments into carbon assimilation via the serine cycle and into the electron transport chain were decreased by all three strains during growth on air compared to the 1000 ppm CH_4 treatment. Overall, the three strains increased the expression of enzymes for the oxidation of at least one trace gas to maximize uptake rates and energy yield during growth on air, while investments in energy conservation and energy-intensive carbon assimilation were decreased.

3.2.3 Specific affinity

To test if atmMOB have a high specific affinity (a_A^0) for CH_4 (expressed by the fraction $V_{max(app)}$ and $K_{m(app)}$) and thus the capacity to efficiently oxidize CH_4 at low concentrations, the a_A^0 of *M. palsarum* NE2 and *M. gorgona* MG08 when grown on air were measured. Additionally, it was tested if the increased investments in pMMO by *M. palsarum* NE2 and the decreased investments by *M. gorgona* MG08 at atmospheric CH_4 concentrations (see section 3.2.2) translate into a higher and lower a_A^0 for CH_4, respectively. The $K_{m(app)}$ of *M. gorgona* MG08 was 48.54 nM CH_4 and the $V_{max(app)}$ 4.91 x 10^{-8} nmol cell^{-1} h^{-1} resulting in a a_A^0 of 1.01 x 10^{-9} L cell^{-1} h^{-1}. The $K_{m(app)}$ of 48.54 nM CH_4 was the first observation of a $K_{m(app)}$ by a methanotroph in pure culture similar to the low $K_{m(app)}$ values of upland soils demonstrating a high apparent affinity for CH_4. The $K_{m(app)}$ of *M. palsarum* NE2 was 402.08 nM CH_4 and the $V_{max(app)}$ 133 x 10^{-8} nmol cell^{-1} h^{-1} resulting in a a_A^0 of 3.30 x 10^{-9} L cell^{-1} h^{-1}. This high a_A^0 suggested that the upregulation of the pMMO at atmospheric CH_4 concentrations in *M. palsarum* NE2 led to a higher CH_4 oxidation capacity compared to *M. gorgona* MG08, which downregulated its pMMO. The results demonstrated that the two atmMOB have a significantly higher a_A^0 for CH_4 than most methanotrophs in pure culture. Additionally, the results demonstrated that a high affinity for CH_4 (expressed by a low $K_{m(app)}$) is not a prerequisite for methanotrophic growth on air, as *M. palsarum* NE2, despite having an eight times higher $K_{m(app)}$, oxidized atmospheric CH_4 quicker than *M. gorgona* MG08. Thus, it was concluded that the a_A^0 is the better model to determine the efficiency of atmospheric CH_4 utilization by atmMOB.

3.2.4 Growth on nitrogen from air

As the atmMOB, *M. gorgona* MG08, *M. palsarum* NE2, *M. rosea* SV97, and *M. aurea* KYG encode all genes required for N_2 fixation and grow on nitrogen-free medium at high CH_4 concentrations, the potential of the four strains to grow with air as sole nitrogen, carbon, and energy source was tested. Filter cultures of all strains formed colonies on air without bioavailable nitrogen source in the medium, suggesting that all strains covered their nitrogen requirements either by N_2 fixation and/or utilization of reactive nitrogen in air. NanoSIMS-based $^{15}N_2$ fixation experiments resulted in cellular ^{15}N enrichments higher than theoretically possible from the $^{15}N_2$ gas used during the experiments. This pointed to reactive nitrogen contamination of the $^{15}N_2$, despite purity test of the gas prior to the experiment resulting in

contamination levels below detection limit. Therefore, N_2 fixation during growth on air could not be confirmed. However, based on the growth on nitrogen-free medium and the NanoSIMS experiments, it was concluded that atmMOB can cover their nitrogen requirements for growth, like energy and carbon, from air.

3.2.5 Conclusions

Major conclusions drawn from the study:

- Growth on air and enduring oxidation of atmospheric CH_4 is not restricted to members of the clades USCα and γ, but more widespread than previously assumed.
- Former liquid culture-based cultivation approaches underestimated the potential of methanotrophic pure cultures to oxidize atmospheric CH_4.
- The strain specific oxidation patterns and the estimations of the a_A^0 for CH_4 demonstrate that both the mixotrophic oxidation of atmospheric trace gases and a high a_A^0 for CH_4 are key to obtain sufficient energy for growth on air.
- Energy requirements for growth of atmMOB are substantially lower than the maintenance energy value used as basic premise for the theory that atmMOB have an oligotrophic lifestyle.
- atmMOB can grow on air by decreasing investments in enzymes involved in energy-intensive processes combined with increasing investments in enzymes for the oxidation of trace gases.
- Differing expression patterns of enzymes for trace gas oxidation and the strain-specific trace gas oxidation patterns indicate that a diverse metabolic repertoire has evolved to enable life on air.
- atmMOB cover not only their energy and carbon but also their nitrogen requirements from the atmosphere.

3.3   Global distrubtion of atmMOB

A
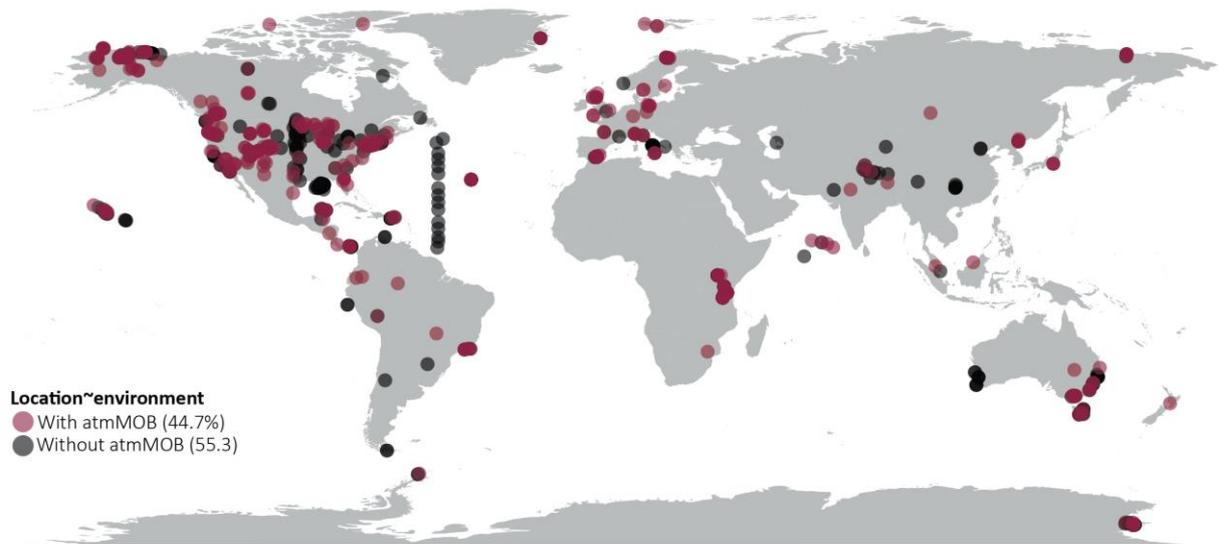
B
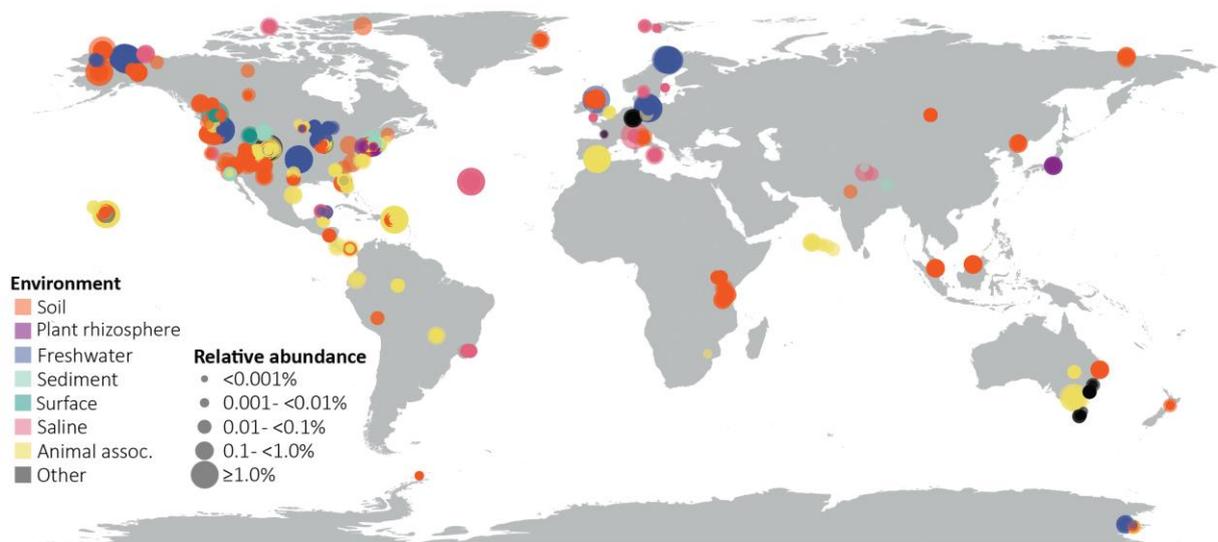
Figure 13. **A)** All geographically unique sample locations and environments accessible in the EPM database and the presence of 16S rRNA gene fragments from putative atmMOB closely related to *M. gorgona, M. aurea, M. palsarum, and M. rosea* within these locations and environments (indicated in red). **B)** Presence and relative abundance of 16S rRNA gene fragments from putative atmMOB closely related to *M. gorgona, M. aurea, M. palsarum, and M. rosea* in EMP samples derived from various environments.

The blast search of the three EMP databases identified 16S rRNA gene fragments of putative atmMOB closely related to *M. gorgona*, *M. aurea*, *M. palsarum, and M. rosea* in 44.7% of all geographically unique sample locations within various climate zones (Figure 13A). The 16S rRNA gene fragments of putative atmMOB were present in non-saline samples from soil, plant

rhizosphere, freshwater, sediments, surfaces (dust, house dust, freshwater biofilms), but also in saline and animal associated samples with abundances ranging from less than 0.001% to more than 1% (Figure 13B).

4 Discussion

The following discussion aims to put the results reported in paper 1 and 2 into a broader scientific context. A thorough discussion of the individual results can be found in the respective papers.

4.1 Physiological strategies of atmMOB to oxidize atmospheric CH$_4$

The finding that "conventional" MOB can temporarily gain the ability to oxidize atmospheric CH$_4$ after exposure to high CH$_4$ concentrations[41,90] demonstrated that flush feeding (ii) (see section 1.6.2) enables a variety of MOB to oxidize atmospheric CH$_4$ for short periods until becoming inactive. However, the discovery of the atmospheric CH$_4$ oxidation by *M. gorgona* MG08 and its genomic potential to oxidize H$_2$ and CO in 2019[101] suggested that a second physiological strategy to oxidize atmospheric CH$_4$ besides flush feeding exists. The simultaneous oxidation of CH$_4$, H$_2$, and CO at their atmospheric trace gas concentration by *M. gorgona* MG08, reported in Paper 1 of this thesis, proofed its metabolic flexibility and mixotrophic nature. This confirmed the hypothetical strategy summarized by Dunfield[32] that facultative methanotrophs (iii) are responsible for oxidation of atmospheric CH$_4$, as *M. gorgona* MG08 consumes other substrates in addition to CH$_4$. However, even though the genetic potential to assimilate CO$_2$ using the reductive glycine pathway (see section 1.10.4) is given, it is unclear if *M. gorgona* MG08 can grow on H$_2$ and CO in absence of CH$_4$. Also, the mixotrophy based on trace gases in air differs from the substrates previously assumed to support survival (e.g., intermediates of aerobic CH$_4$ oxidation or multi carbon compounds like acetate or ethanol) (see section 1.6.3) and enables *M. gorgona* MG08 to utilize the atmosphere as constant and sole source of energy and carbon for growth. Additionally, Paper 1 reported first indications that the energy yield necessary to support cellular maintenance of atmMOB is significantly lower than

assumed in literature[80,85]. This was especially intriguing as the low energy yields from the oxidation of trace gases in air seemed even sufficient for growth. The mixotrophy based on trace gases and these low energy requirements were further investigated in Paper 2 by screening six additional MOB species for their ability to grow with air as sole energy and carbon source. As result, the list of known atmMOB in pure culture able to grow on air was extended by *M. rosea* SV97, *M. palsarum* NE2, and *M. aurea* KYG. Like *M. gorgona* MG08, the three other species also consume atmospheric H_2 and/or CO in addition to CH_4 suggesting that mixotrophy on trace gases might be a common physiological trait of atmMOB able to grow on air. The strongly improved methodology to estimated energy yields from the oxidation of trace gases in air applied in Paper 2 resulted in energy values within the same range of the energy value reported in Paper 1. Thus, not only *M. gorgona* MG08 but also *M. rosea* SV97, *M. palsarum* NE2, and *M. aurea* KYG show energy requirements for growth far below the energy value stated in literature, strongly suggesting that the energy assumed necessary for cellular maintenance of atmMOB has been overestimated. A major problem with this maintenance energy value in literature used to describe the theoretical constraints for microbes utilizing trace gases[80] is that it is not derived from experiments involving atmMOB. It represents the average maintenance energy estimated for a wide range of aerobic and anaerobic microorganisms at different temperatures. The main conclusion of the study reporting this energy value is the correlation of maintenance energy and temperature and not the value itself. Even the authors of the study refer to the energy value as "*first approximation*"[85]. However, due to the absence of atmMOB pure cultures and thus, the possibility to study their energy requirements, the maintenance energy value was used as anchor point to theorize the constrains of microbes utilizing trace gases[80]. Despite their theoretical nature, these constraints that, amongst others, led to the assumption that atmMOB need a high affinity for CH_4 (K_m of 1 pM to 100 nM) for cellular maintenance, were accepted. As consequence, the research field's perspective on how atmMOB survive on air was restricted. For example, a study reported atmospheric CH_4 oxidation rates of the methanotrophic communities in beech and spruce forest soils ranging between 1×10^{-18} and 14×10^{-18} mol CH_4 cell^{-1} h^{-1}. These values are in the range of the CH_4 oxidation rates measured in Paper 2 (2×10^{-18} to 7.3×10^{-18} mol CH_4 cell^{-1} h^{-1}) and too low to meet the maintenance energy value in literature. Instead of questioning the maintenance energy value, the authors of the study concluded that the oxidation rates are *"…lower than required for maintenance of methanotrophic biomass…"* and that methanotrophs of the *"…USCα might*

rely on additional carbon sources to conserve enough energy for cell maintenance and growth[88]. Another example is, as criticized by Dunfield, that the term *"'high-affinity oxidizer' is frequently used interchangeably with 'atmospheric methane oxidizer'…"*[32]. The specific affinity measurements conducted in Paper 2, demonstrated that a high affinity for CH_4 does not necessarily reflect the capability to efficiently oxidize atmospheric CH_4 concentrations. *M. gorgona* MG08 exhibited a high apparent affinity for CH_4 that is within the range of the values theorized for the hypothesis that atmMOB are oligotrophs (see section 1.6.1). However, it oxidized atmospheric CH_4 significantly slower than *M. palsarum* NE2, which exhibited a lower apparent affinity. Taken together, the findings of Paper 1 and 2 strongly suggest to step away from the theorized maintenance energy and affinity assumptions for atmMOB.

The hypothesis that atmMOB are oligotrophs (see section 1.6.1) that can sustain cellular maintenance and growth by only oxidizing atmospheric CH_4 has not been investigated yet. However, considering the low energy values estimated for the four atmMOB studied in Paper 1 and 2 and the predictions that atmospheric CH_4 represents the major energy source for all four strains during growth on air, as shown in Paper 2, it seems likely that atmMOB can live on atmospheric CH_4 as sole energy source.

As the first assessment of the physiological strategies enabling atmMOB in pure culture to grow on air, the findings reported in Paper 1 and 2 revise our understanding of the energy limitation for aerobic growth with major implications for the research field. Considering the omnipresence of atmospheric trace gases, the low energy requirements of atmMOB, and their ability to utilize air as sole carbon and energy source, these organisms could grow in a wide range of surface environments. Additionally, the low energy requirements suggest that the atmosphere as energy source can support the growth of a significantly larger atmMOB population than previously assumed[80]. If true, a higher amount of CH_4 and CO_2 could be sequestered in atmMOB (see section 1.10.4 for carbon assimilation) increasing their impact on the global carbon cycle. Furthermore, if the trace gas based mixotrophy is a common trait of atmMOB, their role in greenhouse gas mitigation, besides the oxidation of atmospheric CH_4, could even be of greater importance as H_2 and CO represent indirect greenhouse gases that deplete the atmospheric OH pool (see section 1.1). Especially the ability to efficiently oxidize H_2 might become valuable since a hydrogen-based economy as part of the industrial decarbonization could lead to increased H_2 emissions[183,184].

4.2   Ecology and phylogeny of atmMOB

Based on the results of Paper 2, the growth on air and the enduring oxidation of atmospheric CH_4 is not restricted to members of the clades USCα and γ, but more widespread than previously assumed. The applied filter cultivation has proven as a suitable tool to assess the capability of MOB to utilize atmospheric trace gases and revealed that the atmMOB *M. palsarum* NE2, *M. rosea* SV97, and *M. aurea* KYG formerly considered as "conventional" methanotrophs can grow on air. Neither their 16S rRNA (see Paper 2) nor their *pmoA* genes[101] cluster within the USCα. This represents a valuable addition to the ecological understanding of atmMOB. On one hand, the diversity of MOB capable to enduringly oxidize atmospheric CH_4 has been underestimated, on the other, a broader range of *pmoA* gene sequences might be indicative for the potential to oxidize atmospheric CH_4. To get a first impression of the ecological relevance of the atmMOB investigated in Paper 2, their global distribution and abundance based on 16S rRNA gene fragments was investigated using EMP databases (see section 2.5 and 3.3). This screening suggested that close relatives of *M. gorgona* MG08, *M. palsarum* NE2, *M. rosea* SV97, and *M. aurea* KYG and thus putative atmMOB are widespread over various climate zones (Fig. 13A) and inhabit not only uplands soils, plants, and caves but are highly abundant in various environments (Fig. 13B). If true, the range of environments contributing to atmospheric CH_4 uptake would be more comprehensive than previously thought as well as the role of atmMOB in carbon cycling and CH_4 mitigation. However, the screening approach used for the global distribution is based on databases containing short 16S rRNA gene fragment sequences (90, 100, and 150 bp). Therefore, the resolution of the results is limited and should be treated with caution as high similarity in 16S rRNA gene fragments does not necessarily translate into the capability to oxidize atmospheric CH_4. To get more reliable results the methodology should base on datasets combing long 16S rRNA sequences and the *pmoA* as functional markers. Nevertheless, the finding in Paper 2 that the atmMOB can utilize the atmosphere as nitrogen source, in addition to carbon and energy, renders the widespread distribution of atmMOB more plausible. Yet, it remains unclear whether the four atmMOB strains can meet their nitrogen requirements by the fixation of N_2 or the assimilation of reactive nitrogen species in the atmosphere or both.

With the rapidly increasing atmospheric CH_4 concentrations and the ongoing depletion of the chemical sink for atmospheric CH_4, (see section 1.1 and 1.3) the importance of the biological sink might increase. However, to investigate and model the response of the biological sink to

the increasing atmospheric CH_4 concentrations and the associated increase in substrate availability, it is crucial to further investigate the diversity and abundance of atmMOB across various environments. Additionally, the impact of other environmental factors on atmMOB (see section 1.5), especially in the context of global warming, needs to be scrutinized. Furthermore, gaining a deeper understanding of how anthropogenic activities, such as practices in agriculture, affect atmMOB is essential to successfully manage or maybe even restore biological sinks for atmospheric CH_4. The four atmMOB pure cultures, along with the methods established throughout this PhD to research them, might be promising steppingstones to do so.

4.3   Metabolic potential of atmMOB for biotechnology

Despite the mixotrophy based on trace gases by all four atmMOB that are capable to grow with air as sole energy and carbon source, their metabolic adjustments to grow on air differ. This is illustrated by the repeatedly measured strain-specific trace gas oxidation patterns reported in Paper 1 and 2. These oxidation patterns were further investigated in Paper 2 that describes the proteome adjustments of atmMOB to grow on air. The results demonstrate that the investigated strains allocated resources to enzymes for the oxidation of trace gases differently. *M. palsarum* NE2 allocated resources towards CH_4 oxidation when grown on air, while *M. gorgona* MG08 and *M. rosea* SV97 allocated resources towards H_2 and CO or H_2 oxidation, respectively. This suggests that the three strains increase their specific affinities for the respective gases on air to maximize trace gas oxidation. The measurements of the specific affinities for CH_4 in Paper 2 are in line with the observed enzyme allocation and show that *M. palsarum* NE2 has the highest specific affinity for CH_4 measured so far. As the specific affinity indicates reaction rates at low substrate concentrations[84], *M. palsarum* NE2 might be an ideal candidate for the biofiltration of emissions containing very low CH_4 concentrations. In contrast to *M. palsarum* NE2, *M. gorgona* MG08 and *M. rosea* SV97 increased their resource allocation towards CH_4 oxidation at "high" CH_4 concentrations (1000 ppm) suggesting high CH_4 oxidation rates at elevated CH_4 concentrations. The enzymatic variability between the strains could enable efficient biofiltration of emission containing a wide range CH_4 concentrations (0.00019 – 5%). This potential for biofiltration of low CH_4 emissions might be of great value as more than 55% of the anthropogenic CH_4 emissions contain CH_4 concentrations below the lower explosive level (LEL) (5% CH_4 in air)[28,185] and thus cannot be combusted for energy recovery. Additionally,

based on its short half-life, the mitigation of CH_4 is considered to be an effective strategy to achieve substantial near-term temperature benefits and to rapidly decelerate global warming[186,187]. Besides the potential of atmMOB to mitigate CH_4 below the LEL, the three strains investigated in detail can utilize the indirect greenhouse gases H_2 and/or CO at atmospheric concentrations. This metabolic flexibility plus their ability to fix CO_2 (see section 1.10.4) might qualify them to efficiently utilize waste gases that contain only minor amounts of CH_4, H_2, or CO as feedstock for biomass production. Thus, atmMOB offer the potential to couple the mitigation of greenhouse gases to the production of compounds with relevance in industry. Examples for methanotrophy-based compounds are, amongst others, byproducts of their metabolism such as methanol or formaldehyde, lipids that could be used as fuel, PHA as precursor for bioplastics, and protein-rich food for humans or animals[13]. In addition, the oligotrophy of the atmMOB, enabling growth with minimal nutrient supply, and their ability to utilize the atmosphere as nitrogen source promises low cultivation costs. Furthermore, by having atmMOB in pure culture, genetic engineering approaches might even optimize their metabolic traits suitable for applications in biotechnology. Overall, especially with regards to the climate crisis, atmMOB appear to offer metabolic traits that could greatly contribute to sustainability.

5   References

1.   IPCC. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Core Writing Team, R.K. Pachauri and L.A. Meyer (2014). doi:10.1017/CBO9781107415324.004.

2.   Intergovernmental Panel on Climate Change. The Earth's Energy Budget, Climate Feedbacks and Climate Sensitivity. in Climate Change 2021 – The Physical Science Basis 923–1054 (Cambridge University Press, 2023). doi:10.1017/9781009157896.009.

3.   Mar, K. A., Unger, C., Walderdorff, L. & Butler, T. Beyond CO2 equivalence: The impacts of methane on climate, ecosystems, and health. Environ Sci Policy **134**, 127–136 (2022).

4.   Reay, D. S., Smith, P., Christensen, T. R., James, R. H. & Clark, H. Methane and Global Environmental Change. Annu Rev Environ Resour **43**, 165–192 (2018).

5.   Lan, X., K.W. Thoning & E.J. Dlugokencky. Trends in globally-averaged CH4, N2O, and SF6 determined from NOAA Global Monitoring Laboratory measurements. Trends in globally-averaged CH4, N2O, and SF6 determined from NOAA Global Monitoring Laboratory measurements (2023) doi:https://doi.org/10.15138/P8XG-AA10.

6.   Kirschke, S. et al. Three decades of global methane sources and sinks. Nature Geoscience vol. 6 813–823 Preprint at https://doi.org/10.1038/ngeo1955 (2013).

7.   Bižić, M. et al. Aquatic and terrestrial cyanobacteria produce methane. Sci Adv **6**, eaax5343 (2020).

8.   Ernst, L. et al. Methane formation driven by reactive oxygen species across all living organisms. Nature 2022 603:7901 **603**, 482–487 (2022).

9.   Saunois, M. et al. The Global Methane Budget 2000–2017. Earth Syst Sci Data **12**, 1561–1623 (2020).

10.   Ehhalt, D. H. The atmospheric cycle of methane. Tellus **26**, 58–70 (1974).

11.   Hanson, R. S. & Hanson, T. E. Methanotrophic Bacteria. MICROBIOLOGICAL REVIEWS vol. 60 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC239451/pdf/600439.pdf (1996).

12.   McGlynn, S. E. Energy Metabolism during Anaerobic Methane Oxidation in ANME Archaea. Microbes Environ **32**, 5–13 (2017).

13.   Guerrero-Cruz, S. et al. Methanotrophs: Discoveries, Environmental Relevance, and a Perspective on Current and Future Applications. Frontiers in Microbiology vol. 12 Preprint at https://doi.org/10.3389/fmicb.2021.678057 (2021).

14.   Valentine, D. L. Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: A review. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology **81**, 271–282 (2002).

15.   Ettwig, K. F. et al. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464**, 543–548 (2010).

16.   Ettwig, K. F. et al. Archaea catalyze iron-dependent anaerobic oxidation of methane. Proc Natl Acad Sci U S A **113**, 12792–12796 (2016).

17.   Haroon, M. F. et al. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. Nature **500**, 567–570 (2013).

18.   Leu, A. O. et al. Anaerobic methane oxidation coupled to manganese reduction by members of the Methanoperedenaceae. ISME Journal **14**, 1030–1041 (2020).

19.   Knief, C. Diversity and Habitat Preferences of Cultivated and Uncultivated Aerobic Methanotrophic Bacteria Evaluated Based on pmoA as Molecular Marker. Front Microbiol **6**, (2015).

20.   Hakobyan, A. & Liesack, W. Unexpected metabolic versatility among type II methanotrophs in the Alphaproteobacteria. Biol Chem **401**, 1469–1477 (2020).

21.   Khadem, A. F. et al. Autotrophic methanotrophy in verrucomicrobia: Methylacidiphilum fumariolicum solv uses the calvin-benson-bassham cycle for Carbon Dioxide Fixation. J Bacteriol **193**, 4438–4446 (2011).

22.   van Spanning, R. J. M. et al. Methanotrophy by a Mycobacterium species that dominates a cave microbial ecosystem. Nat Microbiol **7**, 2089–2100 (2022).

23.   Bay, S. K. et al. Trace gas oxidizers are widespread and active members of soil microbial communities. Nat Microbiol **6**, 246–256 (2021).

24.   Rigby, M. et al. Role of atmospheric oxidation in recent methane growth. Proceedings of the National Academy of Sciences **114**, 5373–5377 (2017).

25.   Jackson, R. B. et al. Increasing anthropogenic methane emissions arise equally from agricultural and fossil fuel sources. Environmental Research Letters **15**, 071002 (2020).

26.   Peng, S. et al. Wetland emission and atmospheric sink changes explain methane growth in 2020. Nature **612**, 477–482 (2022).

27.   Tate, K. R. Soil methane oxidation and land-use change – from process to mitigation. Soil Biol Biochem **80**, 260–272 (2015).

28.   La, H., Hettiaratchi, J. P. A., Achari, G. & Dunfield, P. F. Biofiltration of methane. Bioresour Technol **268**, 759–772 (2018).

29.   Harriss, R. C., Sebacher, D. I. & Day, F. P. Methane flux in the Great Dismal Swamp. Nature **297**, 673–674 (1982).

30.   Bender, M. & Conrad, R. Kinetics of CH4 oxidation in oxic soils exposed to ambient air or high CH4 mixing ratios. FEMS Microbiol Ecol **10**, 261–269 (1992).

31.   Bender, M. & Conrad, R. Kinetics of CH, Oxidation in Oxic Soils Exposed to Ambient Air or High CH, Mixing Ratios. Microbiology Ecology vol. 101 https://academic.oup.com/femsec/article/10/4/261/477403 (1992).

32.   Dunfield, P. F. The soil methane sink. in Greenhouse gas sinks 152–170 (CABI, UK, 2007). doi:10.1079/9781845931896.0152.

33.   Kolb, S. The quest for atmospheric methane oxidizers in forest soils. Environ Microbiol Rep **1**, 336–346 (2009).

34.   Täumer, J. et al. Divergent drivers of the microbial methane sink in temperate forest and grassland soils. Glob Chang Biol **27**, 929–940 (2021).

35.   Seiler, W., Conrad, R. & Scharffe, D. Field studies of methane emission from termite nests into the atmosphere and measurements of methane uptake by tropical soils. J Atmos Chem **1**, 171–186 (1984).

36.   Liu, Y. et al. Atmospheric methane oxidation is affected by grassland type and grazing and negatively correlated to total soil respiration in arid and semiarid grasslands in Inner Mongolia. Soil Biol Biochem **173**, (2022).

37.   Bogner, J., Spokas, K., Burton, E., Sweeney, R. & Corona, V. Landfills as atmospheric methane sources and sinks. Chemosphere **31**, 4119–4130 (1995).

38.   Striegl, R. G., McConnaughey, T. A., Thorstenson, D. C., Weeks, E. P. & Woodward, J. C. Consumption of atmospheric methane by desert soils. Nature **357**, 145–147 (1992).

39.   Kruse, C. W. & Iversen, N. Effect of plant succession, ploughing, and fertilization on the microbiological oxidation of atmospheric methane in a heathland soil. FEMS Microbiol Ecol **18**, 121–128 (1995).

40.   Singh, S., Singh, J. S. & Kashyap, A. K. Methane consumption by soils of dryland rice agriculture: Influence of varieties and N-fertilization. Chemosphere **38**, 175–189 (1999).

41.   Cai, Y., Zheng, Y., Bodelier, P. L. E., Conrad, R. & Jia, Z. Conventional methanotrophs are responsible for atmospheric methane oxidation in paddy soils. Nat Commun **7**, 11728 (2016).

42.   Whalen, S. C. & Reeburgh, W. S. Consumption of atmospheric methane by tundra soils. Nature **346**, 160–162 (1990).

43.   Yun, H. et al. Consumption of atmospheric methane by the Qinghai-Tibet Plateau alpine steppe ecosystem. Cryosphere **12**, 2803–2819 (2018).

44.   Webster, K. D., Mirza, A., Deli, J. M., Sauer, P. E. & Schimmelmann, A. Consumption of atmospheric methane in a limestone cave in Indiana, USA. Chem Geol **443**, 1–9 (2016).

45.   Sundqvist, E., Crill, P., Mlder, M., Vestin, P. & Lindroth, A. Atmospheric methane removal by boreal plants. Geophys Res Lett **39**, (2012).

46.   Liebner, S. et al. Methane oxidation associated with submerged brown mosses reduces methane emissions from Siberian polygonal tundra. Journal of Ecology **99**, 914–922 (2011).

47.   Knief, C., Lipski, A. & Dunfield, P. F. Diversity and Activity of Methanotrophic Bacteria in Different Upland Soils. Appl Environ Microbiol **69**, 6703–6714 (2003).

48.   Holmes, A. J. et al. Characterization of Methanotrophic Bacterial Populations in Soils Showing Atmospheric Methane Uptake. Appl Environ Microbiol **65**, 3312–3318 (1999).

49.   Pratscher, J., Vollmers, J., Wiegand, S., Dumont, M. G. & Kaster, A. Unravelling the Identity, Metabolic Potential and Global Biogeography of the Atmospheric Methane-Oxidizing Upland Soil Cluster α. Environ Microbiol **20**, 1016–1029 (2018).

50.   Cheng, X.-Y. et al. USC γ Dominated Community Composition and Cooccurrence Network of Methanotrophs and Bacteria in Subterranean Karst Caves . Microbiol Spectr (2021) doi:10.1128/SPECTRUM.00820-21.

51.   Täumer, J. et al. Linking transcriptional dynamics of CH4-cycling grassland soil microbiomes to seasonal gas fluxes. ISME J **16**, 1788–1797 (2022).

52.   Rafalska, A., Walkiewicz, A., Osborne, B., Klumpp, K. & Bieganowski, A. Variation in methane uptake by grassland soils in the context of climate change – A review of effects and mechanisms. Science of the Total Environment vol. 871 Preprint at https://doi.org/10.1016/j.scitotenv.2023.162127 (2023).

53.   Wang, Y. et al. Precipitation determines the magnitude and direction of interannual responses of soil respiration to experimental warming. Plant Soil **458**, 75–91 (2021).

54.   King, G. M. & Adamsen, A. P. S. Effects of Temperature on Methane Consumption in a Forest Soil and in Pure Cultures of the Methanotroph Methylomonas rubra. Appl Environ Microbiol **58**, 2758–2763 (1992).

55.   Whalen, StephenC., Reeburgh, WilliamS. & Barber, ValerieA. Oxidation of methane in boreal forest soils: a comparison of seven measures. Biogeochemistry **16**, (1992).

56.   Murguia-Flores, F., Ganesan, A. L., Arndt, S. & Hornibrook, E. R. C. Global Uptake of Atmospheric Methane by Soil From 1900 to 2100. Global Biogeochem Cycles **35**, (2021).

57.   Von Fischer, J. C., Butters, G., Duchateau, P. C., Thelwell, R. J. & Siller, R. In situ measures of methanotroph activity in upland soils: A reactiondiffusion model and field observation of water stress. J Geophys Res Biogeosci **114**, (2009).

58.   Amaral, J. A., Ren, T. & Knowles, R. Atmospheric Methane Consumption by Forest Soils and Extracted Bacteria at Different PH Values. APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 64 (1998).

59.   Kou, Y. et al. Deterministic processes dominate soil methanotrophic community assembly in grassland soils. Geoderma **359**, (2020).

60.   Cai, Y., Zhou, X., Shi, L. & Jia, Z. Atmospheric Methane Oxidizers Are Dominated by Upland Soil Cluster Alpha in 20 Forest Soils of China. Microb Ecol **80**, 859–871 (2020).

61.   Zhao, J., Cai, Y. & Jia, Z. The pH-based ecological coherence of active canonical methanotrophs in paddy soils. Biogeosciences **17**, 1451–1462 (2020).

62.   Fowler, D. et al. The global nitrogen cycle in the Twentyfirst century. Philosophical Transactions of the Royal Society B: Biological Sciences **368**, (2013).

63.   Kanakidou, M. et al. Past, Present, and Future Atmospheric Nitrogen Deposition. (2016) doi:10.1175/JAS-D-15-0278.s1.

64.   Galloway, J. N. et al. Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions. Science (1979) **320**, 889–892 (2008).

65.   Driscoll, C. T. et al. Nitrogen pollution in the northeastern United States: Sources, effects, and management options. BioScience vol. 53 Preprint at https://doi.org/10.1641/0006-3568(2003)053[0357:NPITNU]2.0.CO;2 (2003).

66.   Bodelier, P. L. E. & Steenbergh, A. K. Interactions between methane and the nitrogen cycle in light of climate change. Current Opinion in Environmental Sustainability vols 9–10 26–36 Preprint at https://doi.org/10.1016/j.cosust.2014.07.004 (2014).

67.   Steudler, P. A., Bowden, R. D., Melillo, J. M. & Aber, J. D. Influence of nitrogen fertilization on methane uptake in temperate forest soils. Nature **341**, 314–316 (1989).

68.   Gulledge, J. & Schimel, J. P. Low-Concentration Kinetics of Atmospheric CH_4 Oxidation in Soil and Mechanism of NH_4^+ Inhibition. Appl Environ Microbiol **64**, 4291–4298 (1998).

69.   Bodelier, P. L. E. & Laanbroek, H. J. Nitrogen as a regulatory factor of methane oxidation in soils and sediments. FEMS Microbiology Ecology vol. 47 265–277 Preprint at https://doi.org/10.1016/S0168-6496(03)00304-0 (2004).

70.   Mohanty, S. R., Bodelier, P. L. E., Floris, V. & Conrad, R. Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils. Appl Environ Microbiol **72**, 1346–1354 (2006).

71.   Dam, B., Dam, S., Kim, Y. & Liesack, W. Ammonium induces differential expression of methane and nitrogen metabolism-related genes in Methylocystis sp. strain SC2. Environ Microbiol **16**, 3115–3127 (2014).

72.   Bodelier, P. L. E. & Steenbergh, A. K. Interactions between Methane and Nitrogen Cycling: Current Metagenomic Studies and Future Trends. in.

73.   Shukla, P. N., Pandey, K. D. & Mishra, V. K. Environmental determinants of soil methane oxidation and methanotrophs. Crit Rev Environ Sci Technol **43**, 1945–2011 (2013).

74.   Keller, M., Mitre, M. E. & Stallard, R. F. Consumption of atmospheric methane in soils of central Panama: Effects of agricultural development. Global Biogeochem Cycles **4**, 21–27 (1990).

75.   Knief, C. et al. Diversity of methanotrophic bacteria in tropical upland soils under different land uses. Appl Environ Microbiol **71**, 3826–3831 (2005).

76.   Priemé, A. & Ekelund, F. Five pesticides decreased oxidation of atmospheric methane in a forest soil. Soil Biol Biochem **33**, 831–835 (2001).

77.   Boeckx, P., Van Cleemput, O. & Meyer, T. The influence of land use and pesticides on methane oxidation in some Belgian soils. Biol Fertil Soils **27**, 293–298 (1998).

78.   Yashiro, Y., Kadir, W. R., Okuda, T. & Koizumi, H. The effects of logging on soil greenhouse gas (CO_2, CH_4, N_2O) flux in a tropical rain forest, Peninsular Malaysia. Agric For Meteorol **148**, 799–806 (2008).

79.   Nanba, K. & King, G. M. Response of Atmospheric Methane Consumption by Maine Forest Soils to Exogenous Aluminum Salts †. APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 66 https://journals.asm.org/journal/aem (2000).

80.   Conrad, R. Soil microorganisms oxidizing atmospheric trace gases (CH_4, CO, H_2, NO). Indian J Microbiol **39**, 193–203 (1999).

81.   Novelli, P. C. et al. Molecular hydrogen in the troposphere: Global distribution and budget. Journal of Geophysical Research: Atmospheres **104**, 30427–30444 (1999).

82.   Novelli, P. C., Masarie, K. A. & Lang, P. M. Distributions and recent changes of carbon monoxide in the lower troposphere. Journal of Geophysical Research Atmospheres **103**, 19015–19033 (1998).

83.	Dunfield, P. F. & Conrad, R. Starvation Alters the Apparent Half-Saturation Constant for Methane in the Type II Methanotroph Methylocystis Strain LR1. Appl Environ Microbiol **66**, 4136–4138 (2000).

84.	Button, D. K. Kinetics of nutrient-limited transport and microbial growth. Microbiological Reviews vol. 49 270–297 Preprint at https://doi.org/10.1128/mmbr.49.3.270-297.1985 (1985).

85.	Tijhuis, L., Van Loosdrecht, M. C. M. & Heijnen, J. J. A thermodynamically based correlation for maintenance gibbs energy requirements in aerobic and anaerobic chemotrophic growth. Biotechnol Bioeng **42**, 509–519 (1993).

86.	Knief, C., Kolb, S., Bodelier, P. L. E., Lipski, A. & Dunfield, P. F. The active methanotrophic community in hydromorphic soils changes in response to changing methane concentration. Environ Microbiol **8**, 321–333 (2006).

87.	Kolb, S., Knief, C., Dunfield, P. F. & Conrad, R. Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. Environ Microbiol **7**, 1150–1161 (2005).

88.	Degelmann, D. M., Borken, W., Drake, H. L. & Kolb, S. Different Atmospheric Methane-Oxidizing Communities in European Beech and Norway Spruce Soils. Appl Environ Microbiol **76**, 3228–3235 (2010).

89.	Cai, Y., Zheng, Y., Bodelier, P. L. E., Conrad, R. & Jia, Z. Conventional methanotrophs are responsible for atmospheric methane oxidation in paddy soils. Nat Commun **7**, (2016).

90.	Knief, C. & Dunfield, P. F. Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. Environ Microbiol **7**, 1307–1317 (2005).

91.	Sipkema, E. M., de Koning, W., Ganzeveld, K. J., Janssen, D. B. & Beenackers, A. A. C. M. NADH-regulated metabolic model for growth of Methylosinus trichosporium OB3b. Model presentation, parameter estimation, and model validation. Biotechnol Prog **16**, 176–188 (2000).

92.	Asenjo, J. A. & Suk, J. S. Microbial Conversion of Methane into poly-β-hydroxybutyrate (PHB): Growth and intracellular product accumulation in a type II methanotroph. Journal of Fermentation Technology **64**, 271–278 (1986).

93.	López, J. C., Arnáiz, E., Merchán, L., Lebrero, R. & Muñoz, R. Biogas-based polyhydroxyalkanoates production by Methylocystis hirsuta: A step further in anaerobic digestion biorefineries. Chemical Engineering Journal **333**, 529–536 (2018).

94.	Tveit, A. T. et al. Thermal acclimation of methanotrophs from the genus Methylobacter. The ISME Journal 2023 1–12 (2023) doi:10.1038/s41396-023-01363-7.

95.	Dedysh, S. N., Knief, C. & Dunfield, P. F. Methylocella species are facultatively methanotrophic. J Bacteriol **187**, 4665–4670 (2005).

96. Belova, S. E. et al. Acetate utilization as a survival strategy of peat-inhabiting Methylocystis spp. Environ Microbiol Rep **3**, 36–46 (2011).

97. Bordel, S. et al. Genome scale metabolic modeling reveals the metabolic potential of three Type II methanotrophs of the genus Methylocystis. Metab Eng **54**, 191–199 (2019).

98. Dedysh, S. N. & Dunfield, P. F. Facultative and obligate methanotrophs: How to identify and differentiate Them. in Methods in Enzymology vol. 495 31–44 (Academic Press Inc., 2011).

99. Im, J., Lee, S. W., Yoon, S., Dispirito, A. A. & Semrau, J. D. Characterization of a novel facultative Methylocystis species capable of growth on methane, acetate and ethanol. Environ Microbiol Rep **3**, 174–181 (2011).

100. Hakobyan, A., Zhu, J., Glatter, T., Paczia, N. & Liesack, W. Hydrogen utilization by Methylocystis sp. strain SC2 expands the known metabolic versatility of type IIa methanotrophs. Metab Eng **61**, 181–196 (2020).

101. Tveit, A. T. et al. Widespread soil bacterium that oxidizes atmospheric methane. Proceedings of the National Academy of Sciences **116**, 8515–8524 (2019).

102. Holmes, A. J., Costello, A., Lidstrom, M. E. & Murrell, J. C. Evidence that participate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol Lett **132**, 203–208 (2006).

103. Tavormina, P. L., Orphan, V. J., Kalyuzhnaya, M. G., Jetten, M. S. M. & Klotz, M. G. A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. Environ Microbiol Rep **3**, 91–100 (2011).

104. Su, Y. C., Sathyamoorthy, S. & Chandran, K. Bioaugmented methanol production using ammonia oxidizing bacteria in a continuous flow process. Bioresour Technol **279**, 101–107 (2019).

105. Murrell, J. C. & Dalton, H. Nitrogen Fixation in Obligate Methanotrophs. Microbiology (N Y) **129**, 3481–3486 (1983).

106. Wartiainen, I., Hestnes, A. G., McDonald, I. R. & Svenning, M. M. Methylocystis rosea sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78° N). Int J Syst Evol Microbiol **56**, 541–547 (2006).

107. Dedysh, S. N. et al. Methylocapsa acidiphila gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. Int J Syst Evol Microbiol **52**, 251–261 (2002).

108. Dunfield, P. F., Belova, S. E., Vorob'ev, A. V., Cornish, S. L. & Dedysh, S. N. Methylocapsa aurea sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus Methylocapsa. Int J Syst Evol Microbiol **60**, 2659–2664 (2010).

109.   Dedysh, S. N. et al. Methylocapsa palsarum sp. nov., a methanotroph isolated from a subArctic discontinuous permafrost ecosystem. Int J Syst Evol Microbiol **65**, 3618–3624 (2015).

110.   Inomura, K., Bragg, J. & Follows, M. J. A quantitative analysis of the direct and indirect costs of nitrogen fixation: a model based on Azotobacter vinelandii. The ISME Journal 2017 11:1 **11**, 166–175 (2016).

111.   Whittenbury, R. & Wilkinson, J. F. Enrichment, Isolation and Some Properties of Me thane-utilizing Bacteria. J Gen Microbiol **61**, 205–218 (1970).

112.   Singh, R., Ryu, J. & Kim, S. W. An Overview on Methanotrophs and the Role of Methylosinus trichosporium OB3b for Biotechnological Applications. Biotechnology and Bioprocess Engineering 2022 **27**, 1–14 (2022).

113.   Zhang, T., Zhou, J., Wang, X. & Zhang, Y. Poly-β-hydroxybutyrate Production by Methylosinus trichosporium OB3b at Different Gas-phase Conditions. Iran J Biotechnol **17**, 10–16 (2019).

114.   Belova, S. E. et al. Methylocapsa polymorpha sp. nov., a Novel Dinitrogen-Fixing Methanotroph from a Subarctic Wetland. Microbiology (N Y) **92**, S107–S113 (2023).

115.   Danilova, O. V. et al. One Step Closer to Enigmatic USCα Methanotrophs: Isolation of a Methylocapsa-like Bacterium from a Subarctic Soil. Microorganisms **11**, (2023).

116.   Strong, P. J., Xie, S. & Clarke, W. P. Methane as a resource: Can the methanotrophs add value? Environ Sci Technol **49**, 4001–4018 (2015).

117.   Trotsenko, Y. A. & Murrell, J. C. Metabolic Aspects of Aerobic Obligate Methanotrophy⋆. Adv Appl Microbiol **63**, 183–229 (2008).

118.   Semrau, J. D., Dispirito, A. A. & Yoon, S. Methanotrophs and copper. FEMS Microbiol Rev **34**, 496–531 (2010).

119.   Greening, C. & Grinter, R. Microbial oxidation of atmospheric trace gases. Nat Rev Microbiol **20**, 513–528 (2022).

120.   Koo, C. W., Tucci, F. J., He, Y. & Rosenzweig, A. C. Recovery of particulate methane monooxygenase structure and activity in a lipid bilayer. Science (1979) (2022) doi:10.1126/SCIENCE.ABM3282.

121.   Baba, T. & Miyaji, A. Catalysis and the Mechanism of Methane Conversion to Chemicals: C-C and C-O Bonds Formation Using Heterogeneous, Homogenous, and Biological Catalysts. Catalysis and the Mechanism of Methane Conversion to Chemicals: C-C and C-O Bonds Formation Using Heterogeneous, Homogenous, and Biological Catalysts (Springer Singapore, 2020). doi:10.1007/978-981-15-4132-2.

122.   Miyaji, A., Nitta, M. & Baba, T. Influence of copper ions removal from membrane-bound form of particulate methane monooxygenase from Methylosinus trichosporium OB3b on its activity for methane hydroxylation. J Biotechnol **306**, 100001 (2019).

123.   Shiemke, A. K., Cook, S. A., Miley, T. & Singleton, P. Detergent solubilization of membrane-bound methane monooxygenase requires plastoquinol analogs as electron donors. Arch Biochem Biophys **321**, 421–428 (1995).

124.   Keltjens, J. T., Pol, A., Reimann, J. & Op Den Camp, H. J. M. PQQ-dependent methanol dehydrogenases: Rare-earth elements make a difference. Appl Microbiol Biotechnol **98**, 6163–6183 (2014).

125.   Anthony, C. The quinoprotein dehydrogenases for methanol and glucose. Arch Biochem Biophys **428**, 2–9 (2004).

126.   Chistoserdova, L. Lanthanides: New life metals? World J Microbiol Biotechnol **32**, 1–7 (2016).

127.   Marx, C. J., Chistoserdova, L. & Lidstrom, M. E. Formaldehyde-detoxifying role of the tetrahydromethanopterin-linked pathway in Methylobacterium extorquens AM1. J Bacteriol **185**, 7160–7168 (2003).

128.   Vorholt, J. A. Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. Arch Microbiol **178**, 239–249 (2002).

129.   Kallen, R. G. & Jencks, W. P. The Mechanism of the Condensation of Formaldehyde with Tetrahydrofolic Acid. Journal of Biological Chemistry **241**, 5851–5863 (1966).

130.   Vorholt, J. A., Marx, C. J., Lidstrom, M. E. & Thauer, R. K. Novel Formaldehyde-Activating Enzyme in Methylobacterium Extorquens AM1 Required for Growth on Methanol. JOURNAL OF BACTERIOLOGY vol. 182 https://journals.asm.org/journal/jb (2000).

131.   Vorholt, J. A., Chistoserdova, L., Lidstrom, M. E. & Thauer, R. K. The NADP-dependent methylene tetrahydromethanopterin dehydrogenase in Methylobacterium extorquens AM1. J Bacteriol **180**, 5351–5356 (1998).

132.   Hagemeier, C. H., Chistoserdova, L., Lidstrom, M. E., Thauer, R. K. & Vorholt, J. A. Characterization of a second methylene tetrahydromethanopterin dehydrogenase from Methylobacterium extorquens AM1. Eur J Biochem **267**, 3762–3769 (2000).

133.   Pomper, B. K., Vorholt, J. A., Chistoserdova, L., Lidstrom, M. E. & Thauer, R. K. A methenyl tetrahydromethanopterin cyclohydrolase and a methenyl tetrahydrofolate cyclohydrolase in Methylobacterium extorquens AM1. Eur J Biochem **261**, 475–480 (1999).

134.   Pomper, B. K., Saurel, O., Milon, A. & Vorholt, J. A. Generation of formate by the formyltransferase/hydrolase complex (Fhc) from Methylobacterium extorquens AM1. FEBS Lett **523**, 133–137 (2002).

135.   Moon, M., Park, G. W., Lee, J., Lee, J.-S. & Min, K. Recent progress in formate dehydrogenase (FDH) as a non-photosynthetic CO2 utilizing enzyme: A short review. Journal of CO2 Utilization **42**, 101353 (2020).

136.   Hartmann, T. & Leimkühler, S. The oxygen-tolerant and NAD+-dependent formate dehydrogenase from Rhodobacter capsulatus is able to catalyze the reduction of CO2 to formate. FEBS J **280**, 6083–6096 (2013).

137.   Maden, B. E. H. Tetrahydrofolate and Tetrahydromethanopterin Compared : Functionally Distinct Carriers in C 1 Metabolism. Biochem. J vol. 350 (2000).

138.   Kikuchi, G., Motokawa, Y., Yoshida, T. & Hiraga, K. Glycine cleavage system: reaction mechanism, physiological significance, and hyperglycinemia. Proceedings of the Japan Academy, Series B **84**, 246–263 (2008).

139.   Marx, C. J., Laukel, M., Vorholt, J. A. & Lidstrom, M. E. Purification of the Formate-Tetrahydrofolate Ligase from Methylobacterium extorquens AM1 and Demonstration of Its Requirement for Methylotrophic Growth. J Bacteriol **185**, 7169–7175 (2003).

140.   Lidstrom, M. E. Aerobic Methylotrophic Prokaryotes. The Prokaryotes 618–634 (2006) doi:10.1007/0-387-30742-7_20.

141.   Marx, C. J. & Lidstrom, M. E. Development of an insertional expression vector system for Methylobacterium extorquens AM1 and generation of null mutants lacking mtdA and/or fch. Microbiology (N Y) **150**, 9–19 (2004).

142.   Crowther, G. J., Kosály, G. & Lidstrom, M. E. Formate as the Main Branch Point for Methylotrophic Metabolism in Methylobacterium extorquens AM1. J Bacteriol **190**, 5057–5062 (2008).

143.   Sánchez-Andrea, I. et al. The reductive glycine pathway allows autotrophic growth of Desulfovibrio desulfuricans. Nat Commun **11**, (2020).

144.   Claassens, N. J. Reductive Glycine Pathway: A Versatile Route for One-Carbon Biotech. Trends Biotechnol **39**, 327–329 (2021).

145.   Anthony, C. The Biochemistry of Methylotrophs. (Academic Press, 1982).

146.   Anthony, C. How Half a Century of Research was Required to Understand Bacterial Growth on C1 and C2 Compounds; the Story of the Serine Cycle and the Ethylmalonyl-CoA Pathway. https://doi.org/10.3184/003685011X13044430633960 **94**, 109–137 (2011).

147. Cordero, P. R. F. et al. Atmospheric carbon monoxide oxidation is a widespread mechanism supporting microbial survival. The ISME Journal 2019 13:11 **13**, 2868–2881 (2019).

148. Islam, Z. F. et al. Two Chloroflexi classes independently evolved the ability to persist on atmospheric hydrogen and carbon monoxide. The ISME Journal 2019 13:7 **13**, 1801–1813 (2019).

149. Greening, C., Berney, M., Hards, K., Cook, G. M. & Conrad, R. A soil actinobacterium scavenges atmospheric H2 using two membrane-associated, oxygen-dependent [NiFe] hydrogenases. Proc Natl Acad Sci U S A **111**, 4257–4261 (2014).

150. Schäfer, C. et al. Structure of an Actinobacterial-Type [NiFe]-Hydrogenase Reveals Insight into O 2 -Tolerant H 2 Oxidation. Structure **24**, 285–292 (2016).

151. Dobbek, H., Gremer, L., Kiefersauer, R., Huber, R. & Meyer, O. Catalysis at a dinuclear [CuSMo(=O)OH] cluster in a CO dehydrogenase resolved at 1.1-Å resolution. Proc Natl Acad Sci U S A **99**, 15971–15976 (2002).

152. Greening, C., Berney, M., Hards, K., Cook, G. M. & Conrad, R. A soil actinobacterium scavenges atmospheric H 2 using two membrane-associated, oxygen-dependent [NiFe] hydrogenases. Proceedings of the National Academy of Sciences **111**, 4257–4261 (2014).

153. Cordero, P. R. F. et al. Two uptake hydrogenases differentially interact with the aerobic respiratory chain during mycobacterial growth and persistence. Journal of Biological Chemistry **294**, 18980–18991 (2019).

154. Rohde, M., Mayer, F. & Meyer, O. Immunocytochemical localization of carbon monoxide oxidase in Pseudomonas carboxydovorans. The enzyme is attached to the inner aspect of the cytoplasmic membrane. Journal of Biological Chemistry **259**, 14788–14792 (1984).

155. Grinter, R. et al. Structural basis for bacterial energy extraction from atmospheric hydrogen. Nature 2023 615:7952 **615**, 541–547 (2023).

156. Auman, A. J., Speake, C. C. & Lidstrom, M. E. nifH Sequences and Nitrogen Fixation in Type I and Type II Methanotrophs. Appl Environ Microbiol **67**, 4009–4016 (2001).

157. Khadem, A. F., Pol, A., Jetten, M. S. M. & Op den Camp, H. J. M. Nitrogen fixation by the verrucomicrobial methanotroph 'Methylacidiphilum fumariolicum' SolV. Microbiology (N Y) **156**, 1052–1059 (2010).

158. Hara, S. et al. In Vivo Evidence of Single 13C and 15N Isotope–Labeled Methanotrophic Nitrogen-Fixing Bacterial Cells in Rice Roots. mBio **13**, (2022).

159. Raymond, J., Siefert, J. L., Staples, C. R. & Blankenship, R. E. The Natural History of Nitrogen Fixation. Mol Biol Evol **21**, 541–554 (2004).

160.   Seefeldt, L. C. et al. Reduction of Substrates by Nitrogenases. Chemical Reviews vol. 120 5082–5106 Preprint at https://doi.org/10.1021/acs.chemrev.9b00556 (2020).

161.   Poole, R. K. & Hill, S. Respiratory Protection of Nitrogenase Activity in Azotobacter vinelandii —Roles of the Terminal Oxidases. Biosci Rep **17**, 303–317 (1997).

162.   Gallon, J. R. Reconciling the incompatible: N2 fixation And O2. New Phytologist **122**, 571–609 (1992).

163.   Gebhardt, C., Turner, G. L., Gibson, A. H., Dreyfus, B. L. & Bergersen, F. J. Nitrogen-fixing Growth in Continuous Culture of a Strain of Rhizobium sp. Isolated from Stem Nodules on Sesbania rostrata. Microbiology (N Y) **130**, 843–848 (1984).

164.   Fay, P. Oxygen Relations of Nitrogen Fixation in Cyanobacteria. MICROBIOLOGICAL REVIEWS vol. 56 https://journals.asm.org/journal/mr (1992).

165.   Schlesier, J., Rohde, M., Gerhardt, S. & Einsle, O. A Conformational Switch Triggers Nitrogenase Protection from Oxygen Damage by Shethna Protein II (FeSII). J Am Chem Soc **138**, 239–247 (2016).

166.   Threatt, S. D. & Rees, D. C. Biological nitrogen fixation in theory, practice, and reality: a perspective on the molybdenum nitrogenase system. FEBS Letters vol. 597 45–58 Preprint at https://doi.org/10.1002/1873-3468.14534 (2023).

167.   Kalescky, R., Kraka, E. & Cremer, D. Identification of the strongest bonds in chemistry. in Journal of Physical Chemistry A vol. 117 8981–8995 (2013).

168.   Roberts, G. P., Macneil, T., Macneil, D. & Brill, W. J. Regulation and Characterization of Protein Products Coded by the Nif (Nitrogen Fixation) Genes of Klebsiella Pneumoniae. JOURNAL OF BACTERIOLOGY https://journals.asm.org/journal/jb (1978).

169.   Lukoyanov, D. et al. Reductive Elimination of H2 Activates Nitrogenase to Reduce the N≡N Triple Bond: Characterization of the E4(4H) Janus Intermediate in Wild-Type Enzyme. J Am Chem Soc **138**, 10674–10683 (2016).

170.   Ferrari, B. C., Binnerup, S. J. & Gillings, M. Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. Appl Environ Microbiol **71**, 8714–8720 (2005).

171.   Svenning, M. M., Wartiainen, I., Hestnes, A. G. & Binnerup, S. J. Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system. FEMS Microbiol Ecol **44**, 347–354 (2003).

172.   Burg, T. P. et al. Weighing of biomolecules, single cells and single nanoparticles in fluid. Nature **446**, 1066–1069 (2007).

173.   Grover, W. H. et al. Measuring single-cell density. Proceedings of the National Academy of Sciences **108**, 10992–10996 (2011).

174.   Feijó Delgado, F. et al. Intracellular Water Exchange for Measuring the Dry Mass, Water Mass and Changes in Chemical Composition of Living Cells. PLoS One **8**, e67590 (2013).

175.   Lechene, C. et al. High-Resolution Quantitative Imaging of Mammalian and Bacterial Cells Using Stable Isotope Mass Spectrometry. http://jbiol.com/content/5/6/20 (2006).

176.   Herrmann, A. M. et al. Nano-scale secondary ion mass spectrometry - A new analytical tool in biogeochemistry and soil ecology: A review article. Soil Biol Biochem **39**, 1835–1850 (2007).

177.   Deracinois, B., Flahaut, C., Duban-Deweer, S. & Karamanos, Y. Comparative and quantitative global proteomics approaches: An overview. Proteomes vol. 1 180–218 Preprint at https://doi.org/10.3390/proteomes1030180 (2013).

178.   Thompson, L. R. et al. A communal catalogue reveals Earth's multiscale microbial diversity. Nature **551**, 457–463 (2017).

179.   Gilbert, J. A., Jansson, J. K. & Knight, R. The Earth Microbiome project: Successes and aspirations. BMC Biology vol. 12 Preprint at https://doi.org/10.1186/s12915-014-0069-1 (2014).

180.   Gilbert, J. A. et al. Meeting Report: The Terabase Metagenomics Workshop and the Vision of an Earth Microbiome Project. Stand Genomic Sci **3**, 243–248 (2010).

181.   Camacho, C. et al. BLAST+: Architecture and applications. BMC Bioinformatics **10**, (2009).

182.   R Core Team. R: A Language and Environment for Statistical Computing. Preprint at https://www.R-project.org/ (2022).

183.   Ocko, I. B. & Hamburg, S. P. Climate consequences of hydrogen emissions. Atmos Chem Phys **22**, 9349–9368 (2022).

184.   Dincer, I. Hydrogen 1.0: A new age. International Journal of Hydrogen Energy vol. 48 16143–16147 Preprint at https://doi.org/10.1016/j.ijhydene.2023.01.124 (2023).

185.   Avalos Ramirez, A., García-Aguilar, B. P., Jones, J. P. & Heitz, M. Improvement of methane biofiltration by the addition of non-ionic surfactants to biofilters packed with inert materials. Process Biochemistry **47**, 76–82 (2012).

186.   Smith, S. J. et al. Impact of methane and black carbon mitigation on forcing and temperature: a multi-model scenario analysis. Clim Change **163**, 1427–1442 (2020).

187.   Ocko, I. B. et al. Acting rapidly to deploy readily available methane mitigation measures by sector can immediately slow global warming. Environmental Research Letters **16**, (2021).

Publications

Paper 1

Article

Simultaneous Oxidation of Atmospheric Methane, Carbon Monoxide and Hydrogen for Bacterial Growth

Alexander Tøsdal Tveit [1,*,†] **, Tilman Schmider** [1,†]**, Anne Grethe Hestnes** [1]**, Matteus Lindgren** [2]**, Alena Didriksen** [1] **and Mette Marianne Svenning** [1]

1 Department of Arctic and Marine Biology, UiT, The Arctic University of Norway, 9037 Tromsø, Norway; tilman.schmider@uit.no (T.S.); anne.hestnes@uit.no (A.G.H.); alena.didriksen@uit.no (A.D.); mette.svenning@uit.no (M.M.S.)

2 CAGE—Centre for Arctic Gas Hydrate, Environment and Climate, Department of Geosciences, UiT, The Arctic University of Norway, 9010 Tromsø, Norway; matteus.lindgren@uit.no

* Correspondence: alexander.t.tveit@uit.no

† These authors contributed equally to this work.

Abstract: The second largest sink for atmospheric methane (CH_4) is atmospheric methane oxidizing-bacteria (atmMOB). How atmMOB are able to sustain life on the low CH_4 concentrations in air is unknown. Here, we show that during growth, with air as its only source for energy and carbon, the recently isolated atmospheric methane-oxidizer *Methylocapsa gorgona* MG08 (USCα) oxidizes three atmospheric energy sources: CH_4, carbon monoxide (CO), and hydrogen (H_2) to support growth. The cell-specific CH_4 oxidation rate of *M. gorgona* MG08 was estimated at ~0.7×10^{-18} mol cell^{-1} h^{-1}, which, together with the oxidation of CO and H_2, supplies 0.38 kJ Cmol^{-1} h^{-1} during growth in air. This is seven times lower than previously assumed necessary to support bacterial maintenance. We conclude that atmospheric methane-oxidation is supported by a metabolic flexibility that enables the simultaneous harvest of CH_4, H_2 and CO from air, but the key characteristic of atmospheric CH_4 oxidizing bacteria might be very low energy requirements.

Keywords: methane; carbon monoxide; hydrogen; energy; growth; atmospheric trace gases

1. Introduction

Atmospheric methane-oxidizing bacteria (atmMOB) remove 9–47 Tg methane (CH_4) from the atmosphere annually [1]. The most common hypothesis for explaining how these bacteria can sustain life by oxidizing atmospheric CH_4 is the "high affinity" model, in which a special form of particulate CH_4 monooxygenase allows atmMOB to oxidize the low atmospheric CH_4 concentrations at a high rate [2]. Other hypotheses include the utilization of alternative energy sources or a high specific affinity ($V_{max(app)}/K_{m(app)}$) [3,4]. AtmMOB are found globally in soils [4] and belong to the two phylogenetic clusters USCα (Alphaproteobacteria) and USCγ (Gammaproteobacteria) [5]. The lack of pure cultures from USCα and USCγ has prevented studies of the energy metabolism of atmMOB [3]. Conventional methane oxidizing bacteria (MOB) are expected to oxidize around $0.2–17 \times 10^{-18}$ mol CH_4 cell^{-1} h^{-1} at atmospheric CH_4 concentrations at 25 °C, but such rates have been considered too low to provide sufficient energy for growth [6]. Thus, atmMOB were expected to either utilize alternative energy sources or have high affinities for CH_4 [3]. The latter is supported by observations reporting apparent high affinity CH_4 oxidation by microbial communities in oxic soils [7]. Additionally, oxidation rates of 800×10^{-18} mol CH_4 cell^{-1} h^{-1} were measured in forest soils, far surpassing the cell-specific activity of 40×10^{-18} mol CH_4 cell^{-1} h^{-1} assumed to be necessary to support minimum cell maintenance requirements [6,8]. At 20 °C, these minimum requirements would demand an energy supply of 2.8 kJ Cmol h^{-1}. It is assumed that the high forest soil rates at atmospheric CH_4 concentrations are achieved by the combination of methane

monooxygenases (MMO) with very low half-saturation constants (K_m) and high cellular abundances of these enzymes.

The recently described USCα atmMOB species *Methylocapsa gorgona* MG08 has the highest specific affinity ($V_{\mathrm{max(app)}}/K_{m(app)}$) for CH_4 of all tested MOB at 195×10^{-12} L·cell^{-1}·h^{-1}. However, this translates into a cell-specific CH_4 oxidation rate of only 10×10^{-18} mol CH_4 cell^{-1} h^{-1} at atmospheric CH_4 concentration [4], seemingly too low to support cellular maintenance [6]. Nevertheless, *M. gorgona* MG08 is able to grow at atmospheric (1.87 p.p.m.v.) CH_4 concentrations [4]. It also carries a [NiFe] group 1 h high-affinity respiratory hydrogenase (*hhyL* and *hhyS*) and a [MoCu] class I respiratory carbon monoxide dehydrogenase, similar to those identified in atmospheric carbon monoxide (CO) and hydrogen (H_2)-oxidizing microorganisms [4,9–12]. Recent studies have identified the utilization of atmospheric CO and H_2 as energy sources for growth and survival in bacteria [9,11–13], and as support for bacterial primary production in Antarctic and Arctic environments [10,14]. Atmospheric CH_4 concentrations are currently 1.87 p.p.m.v. [15], while the concentrations of atmospheric CO and H_2 are lower. CO concentrations vary, with estimates for uninhabited areas around 0.1 p.p.m.v., while urban areas contain higher concentrations, often above 0.2 p.p.m.v. [16–19]. Atmospheric H_2 concentrations are stable at approximately 0.53 p.p.m.v. [20]. Many MOB carry genes for carbon monoxide and hydrogen oxidation [4], and recently it has been shown that the thermoacidophilic *Methylacidiphilum fumariolicum* SolV can oxidize sub-atmospheric H_2 with a high-affinity, membrane-associated [NiFe] hydrogenase [21], while the strain *Methylocystis* sp. SC2 oxidizes hydrogen at higher concentrations [22]. However, the oxidation of atmospheric CO and H_2 to support growth has never been demonstrated for *M. gorgona* MG08 or any other MOB. We have studied how the atmospheric CH_4 oxidizer *M. gorgona* MG08, in pure culture, harvests energy from the atmosphere for growth.

2. Materials and Methods

2.1. Cultivation

A young stationary culture of *M. gorgona* MG08 was prepared as follows. A 20 mL culture (1:10 (NMS:MilliQ) in diluted liquid nitrate mineral salts (NMS) medium (pH 6.8) (DSMZ medium 921) without EDTA) with a headspace of 20% CH_4 in air (20 mL 100% CH4 mixed with 80 mL of air) was incubated for 12 days, reaching several days (2–4) into the stationary phase. Inoculum from this culture was precultured in 1:10 (NMS:MilliQ) diluted liquid nitrate mineral salts (NMS) medium (pH 6.8) (DSMZ medium 921) without EDTA under a headspace atmosphere of ~20 p.p.m.v. CH_4 in air for two weeks. The headspace was created by injecting 20 μL of 100% CH_4 into the 100 mL ambient air headspace of 120 mL serum bottles containing 20 mL medium, sealed with a butyl rubber stopper and crimp cap. This was used as a start culture for colony growth on filters. The cell concentration in the suspension was determined by fluorescence microscopy after filtration on Anodisc filters (Whatman 6809-6022, Merck, Darmstadt, Germany) and 1000× SYBRgreen I (Molecular probes S-7567, ThermoFisher, Waltham, MA, USA) staining as previously described [4]. For staining, filters were transferred (bacteria side up) on top of 200 μL droplets of 1000× SYBRgreen and incubated for 10 minutes. In the next step, filters were washed twice by transferring them onto 1 mL milliQ water and then air-dried. The whole procedure was performed at room temperature and in the dark. A fresh-made anti-fading solution consisting of 0.1% p-phenylenediamine dihydrochloride in 1:1 glycerol and PBS (phosphate buffered saline pH 7.2) was used for mounting the filters on slides with cover slips.

From the start cultures, the required volume and cell density needed to achieve a cell density of ~20 cells per 63× photo area, for a total of ~1×10^6 cells per filter, was selected for filter cultivation. For the transfer of cells onto the 47 mm polycarbonate filter, an autoclaved Millipore vacuum filter holder system (cat no XX1004700, Merck, Darmstadt, Germany) with 300 mL glass funnels was used. Firstly, a GF/C filter (Whatman 1822-047, Merck, Darmstadt, Germany) was added as support filter. A polycarbonate filter

(Whatman, Nucleopore 111106, Merck, Darmstadt, Germany) was then added and the funnel was clamped on top. Even distributions of cells on the filters were obtained by pouring 100 mL of autoclaved milliQ-water into the funnel before mixing in 0.1 mL of the start culture cell suspension and applying vacuum. The funnel walls were rinsed twice with 15 mL sterile water during suction. The polycarbonate filters were then transferred into 250 mL bottles (DURAN, Merck, Darmstadt, Germany) in a Holten LaminAir bench and left floating on 50 mL of 1:10 (NMS:MilliQ) diluted liquid NMS medium (pH 6.8) without EDTA. Finally, the bottles were covered with gas permeable parafilm (Merck, Darmstadt, Germany). These bottles were incubated in a ventilated room at 20 °C in darkness for nine months (experiment 1) or 4.5 months (experiment 2), after which the gas uptake experiments were initiated. In Experiment 1, an additional inspection of a filter was performed after eight months. This included a visual inspection and counting of 60 colonies on two different filters to show that the number of generations in almost all growing colonies had surpassed seven (64 cells), indicating that the filters had sufficient biomass to initiate gas uptake experiments.

2.2. Temperature Selection

All cultivations and experiments were carried out at 20 °C. This temperature was chosen as it is in the middle of the optimum temperature range for growth (~15–~27 °C) of *M. gorgona* MG08 and was the temperature at which previous experiments were carried out [4].

2.3. Gas Uptake and Leakage Experiments

Three experiments were carried out: two gas uptake experiments that included controls and one control experiment. Experiment 1 consisted of 53 × 120 mL bottles, some containing cultures of *M. gorgona* MG08 on filters floating on liquid media. Experiment 2 consisted of 15 bottles. The control experiment consisted of 18 bottles.

In all experiments, the bottles contained 50 mL of 1/10 diluted NMS medium without EDTA. All gases used were of the highest available quality (6.0) (all gases were supplied by AGA). For the first gas uptake experiment, a total of 7 different setups (A–G) of five replicates each were created: A–E contained filters with *M. gorgona* MG08 cells, and F and G did not contain *M. gorgona* MG08 cells. (A) five bottles with 2–4 p.p.m.v. CH_4, 2–4 p.p.m.v. H_2 and 2–4 p.p.m.v. CO in synthetic air. (B) five bottles with 2–4 p.p.m.v. H_2 and 2–4 p.p.m.v. CO in synthetic air; (C) five bottles with 2–4 p.p.m.v. CH_4 in synthetic air. (D) five bottles with an atmosphere of ambient air (compressed outdoor air); (E) five bottles with synthetic air. (F) five bottles with 2–4 p.p.m.v. CH_4, 2–4 p.p.m.v. H_2 and 2–4 p.p.m.v. CO in synthetic air. (G) five bottles with ambient air atmosphere.

For the second gas uptake experiment, one condition included cells; five bottles with ~4 p.p.m.v. CH_4, ~4 p.p.m.v. H_2 and ~4 p.p.m.v. CO in synthetic air. Two conditions included sterile filters; five bottles with ~4 p.p.m.v. CH_4, ~4 p.p.m.v. H_2 and ~4 p.p.m.v. CO in synthetic air and five bottles with synthetic air atmospheres.

The control experiment to determine gas leakages (18 bottles) included six different types of liquids and headspace compositions: (A) 50 mL MilliQ water and 200 mL headspace of pure helium. (B) Sterile floating polycarbonate filter on 50 mL of 1/10 diluted NMS medium without EDTA and 200 mL headspace with 2–5 p.p.m.v. CH_4, H_2, CO in synthetic air. (C) 50 mL of milliQ water and synthetic air. (D) 50 mL of 1/10 diluted NMS medium without EDTA and 2–5 p.p.m.v. CH_4, H_2, CO in synthetic air. (E) 50 mL of milliQ water and 2–5 p.p.m.v. CH_4, H_2, CO in synthetic air. (F) Empty glass bottle with 250 mL of 2–5 p.p.m.v. CH_4, H_2, CO in synthetic air.

To create the mentioned atmospheres, the bottles were sealed with halogenated bromobutyl rubber stoppers (DURAN, Merck, Darmstadt, Germany) and plastic screw caps under a sterile bench. Before usage, the rubber stoppers had been boiled ten times and then autoclaved. The sealed bottles were flushed for 15 min with ambient, high quality synthetic air (N_2: 78%, O_2: 21%, CO_2: 400 p.p.m.v.) or pure helium by using a gassing manifold

connected to sterile single-use needles. The final pressure in the bottles was adjusted to one bar absolute pressure. Afterwards, 1 mL of additional respective gases (CH_4, CO and H_2) was added by using a gas tight syringe (VICI AG International, Schenkon, Switzerland) to create the required atmospheres. To prevent contamination, each gassing step was carried out by interconnecting a sterile 0.2 μm cellulose acetate filter (VWR Collection, Lutterworth, UK) between needle and hose or syringe.

The prepared bottles were incubated at 20 °C for 57 days in the first experiment. The headspace concentrations of CH_4, CO and H_2 were measured the day after preparing the headspace atmospheres and after 7, 17 and 57 days. In the second experiment, the incubation lasted 145 h. In the control experiment, the incubations lasted 6, 54 or 70 days.

For combined measurements of CH_4, CO and H_2, 1 mL headspace gas was sampled with a gastight GC syringe (VICI AG International, Schenkon, Switzerland). The contained gas was injected manually into a gas chromatograph (ThermoScientific Trace 1310 with column TG-BOND Msieve 5A, ThermoFisher, Waltham, MA, USA). Detection was achieved by using a PDD detector. A high-quality gas containing 5 p.p.m.v. H_2, 5 p.p.m.v. CH_4, and 5 p.p.m.v. CO in N_2 served as standard. To create standard curves, 2×0.1 mL, 2×0.5 mL, and 2×1 mL of the mentioned standard were injected on every measurement day. Bottle gas concentrations were calculated using the standard curve. Masses were calculated by applying the ideal gas law and adjusted for changes in bottle pressure due to gas removal.

2.4. Cell Quantification

Due to several layers of cells forming colonies on the filters after 9 months and 57 days of incubation, the number of cells on four filters per condition A–E in the gas uptake experiment could not be estimated reliably by cell counts. Instead, cell numbers were estimated using DNA extractions and comparison to a standard of DNA extraction yields for known cell numbers of *M. gorgona* MG08 on polycarbonate filters. Cells for a standard were prepared as follows. A 20 mL culture (1:10 (NMS:MilliQ) diluted liquid nitrate mineral salts (NMS) medium (pH 6.8) (DSMZ medium 921) without EDTA) with a headspace of 20% CH_4 in air (20 mL 100% CH_4 mixed with 80 mL of air) was incubated for 12 days, reaching several days (2–4) into the stationary phase. The culture was then exposed to atmospheric concentrations of CH_4 for one additional day during which the number of cells in the culture was estimated using cell counts. Then, the cells were filtered out and the filters were prepared for extractions. With this approach, our aim was to extract DNA from cells in a slow growth state where the cells do not contain multiple partial genomes. Filters for the standard and those from the experiment incubation were cut into 12 pieces and put into 2 mL safelock tubes, after which DNA was extracted from the filters using DNA IQ™ Casework Pro Kit for Maxwell® 16 following the manufacturer's instructions. In brief, we crushed the 12 filter pieces with a metal bead in a tissue lyser (Qiagen, Hilden, Germany) six times, 30 s each, applying liquid nitrogen prior to the first and between each round in the tissue lyser. Prior to extraction, the crushed pieces were spun down. After, 400 μL of extraction buffer containing proteinase K and thioglycerol followed by 400 μL lysis buffer was added. Then, the mixture was transferred into a cartridge of the Maxwell 16 system and processed. DNA extracts were quantified using Qubit (dsDNA HS Assay, Thermo Fisher, Waltham, MA, USA). Cell numbers on filters from the experiment were subsequently determined by comparing the extract yields per filter to those of the standard, converting the values from nanograms of DNA to cell numbers.

2.5. Contamination Tests and Microscopy

In addition to the start of the pre-incubation and after eight months, we inspected filters by fluorescence microscopy at the beginning and end of the first gas uptake experiment. At the end of the experiment, one filter from each of the five treatments was used for contamination tests and fluorescence microscopy. For the second gas uptake experiment, we inspected filters at the beginning and end of the pre-incubation after 4.5 months, in addition to contamination tests after the experiment. Preparation for fluorescence mi-

croscopy was carried out as described for the pre-incubation above. Contamination tests were carried out by cutting filters in pieces and placing them on TGYA plates for 15 days. TGYA plates contained 5 g tryptone, 2.5 g of yeast extract, 1 g of glucose, and 20 g of agar per 1 L of water. Presence and growth of heterotrophic microorganisms was then evaluated by visual inspection. The contamination tests were negative and cell sizes and cell shapes were the same as prior to the experiment, confirming the purity of the culture.

2.6. Plotting and Statistics

All plotting was performed using the R [23] package ggplot.2 [24].

2.7. Cell-Specific Oxidation Rates and Free Energy Yield Calculations

Oxidation rates were estimated by first order rate kinetics models. We log transformed the concentrations over time and fitted linear regression models to the transformed plots. The slope of the linear models corresponds to the rate constants. By multiplying the respective rate constant by the atmospheric concentrations of CH_4, H_2 and CO, we obtained the rates of oxidation at atmospheric gas concentrations. The fit of the linear models was evaluated and considered to have satisfactory coefficients of determination, giving the following values: $R^2 - CH_4 = 0.93$, $R^2 - H_2 = 0.82$, $R^2 - CO = 0.68$ (Experiment 1), and $R^2 - CH_4 = 0.99$, $R^2 - H_2 = 0.99$, $R^2 - CO = 0.99$ (Experiment 2). The oxidation rates of H_2 and CO were subsequently corrected by adjusting for the abiotic gas leakages. Due to the first order rate kinetics nature of the CO (R^2 of linear models = 0.77, $n = 5$) and H_2 (R^2 of linear models = 0.99, $n = 5$) leakages, their respective rate constants were estimated the same way as for the oxidation rates. The rate constants for the biological H_2 and CO oxidation were subsequently adjusted by subtracting or adding the rate constants of the leaks of H_2 (leakage out of the bottle) and CO (leakage into), respectively. For each condition A, B, C, D and E, cell numbers on three filters were estimated. Cell-specific oxidation rates were subsequently calculated by dividing the estimated oxidation rates by the corresponding cell numbers.

The expected maintenance requirements at 20 °C were calculated according to Tijhuis et al. [8]. The Gibbs free energy changes ($\Delta_r G$) were calculated for the following reactions and Gibbs free energies of formation $\Delta G_f°$ (kJ/mol), assuming atmospheric concentrations and 20 °C: $[CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O]$; $[2CO + O_2 \rightarrow 2CO_2]$; $[2H_2 + O_2 \rightarrow 2H_2O]$. CO: -137.16 kJ/mol, O_2: 0 kJ/mol, CO_2: -394.39 kJ/mol, H_2: 0 kJ/mol, H_2O: -237.13 kJ/mol, CH_4: -50.6 kJ/mol. From the oxidation rates and free energy change of the reactions, we could estimate the amount of energy obtained per mol of biomass carbon per hour, applying the dry weight and carbon content of *M. gorgona* MG08 (see below) as previously shown [2,6]. All calculations, raw data and literature data needed for input in the calculations are provided in a detailed format. The data and calculations are provided as excel formulas that can be intuitively followed and inspected. These can serve as a template for those wishing to repeat our calculations for similar experiments and compare those to our data (See Supplementary dataset Table S1, tabs "Experiment 1", "Experiment 2", "Gibbs_free_energy" and "Maintenance_energy").

2.8. Estimating Cell Dry Weight

In order to estimate the dry weight of *M. gorgona* MG08, we cultivated 10×10 mL of *M. gorgona* MG08 culture as described for a young stationary culture in the cultivation section above. This provided ten replicates from which 1 mL was used for cell counts and 9 mL for drying overnight at 110 °C. Based on this, we calculated that the weight of one *M. gorgona* MG08 cell is 8.8×10^{-14} g dry weight per cell (SD = 3.1×10^{-14}). We assume that half of the cell consists of carbon [2]. This weight and size (length 1.2 μm and width 0.7 μm) makes *M. gorgona* MG08 the lightest known Alphaproteobacteria MOB, compared to previously weighed strains [2]. While the size of *M. gorgona* MG08 cells do not change when growing with air as its energy and carbon source, compared to higher CH_4 concentrations (it retains its size of length ~1.2 μm and width ~0.7 μm), we acknowledge

that the content, density and thus dry weight of the cells growing in air may differ from that of *M. gorgona* MG08 cultivated at high CH_4 concentrations. Such differences could affect the estimates of kJ Cmol h^{-1}. However, due to the low biomass, we were not able to estimate cell weights from filters cultivated in air.

3. Results and Discussion

Cells of *M. gorgona* MG08 were pre-incubated on filters under an atmosphere of ambient air (~1.84 p.p.m.v. CH_4, ~0.39 p.p.m.v. CO and ~0.7 p.p.m.v. H_2) for eight months. The filters floated on diluted mineral medium within bottles to enable optimal gas transfer. After four months, a subset of the cells had developed into microcolonies of more than 100 cells (Figure 1), while some cells had not divided. Visual inspection and counting showed that the number of generations in the majority of the colonies had surpassed seven (64 cells) (Figure 1C), matching previous observations [4]. Studies have shown that up to three generations can be supported by intracellular storages [4,25,26], confirming that the eight-month pre-incubation was sufficient to ensure growth with carbon and energy harvested solely from air. After nine months of pre-incubation, the filter-containing glass bottles were sealed with rubber stoppers before defined headspace atmospheres were created (Figure 2).
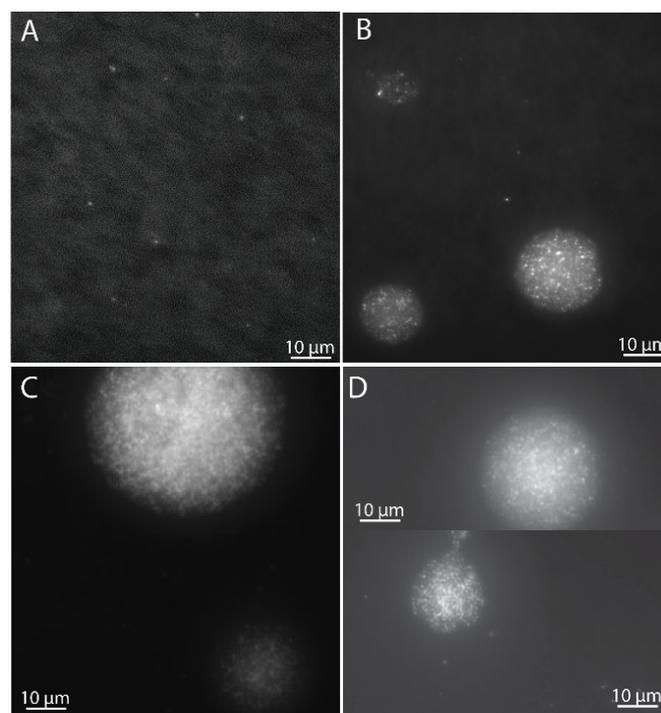
Figure 1. Microcolony growth of *M. gorgona* MG08 at different times during pre-incubation and gas uptake experiments. (**A**) Cells after filtration on polycarbonate filters, prior to incubation. (**B**) Microcolonies after 4 months of pre-incubation under ambient air. (**C**) Microcolonies after 8 months of pre-incubation under ambient air. (**D**) Microcolonies after 9 months of pre-incubation under ambient air and 57 days of gas uptake experiment incubation under synthetic air ((**D**), upper panel), and synthetic air + 2–4 p.p.m.v. CH_4, CO and H_2 ((**D**), lower panel). For fixation, the filters were transferred to fresh-made 2% paraformaldehyde in 1× PBS in the refrigerator overnight. For staining, filters were transferred (side with bacteria up) on top of 200 µL droplets of 1000× SYBRgreen I (10× dilution of the stock concentration provided by Thermo Fisher Scientific, Invitrogen, Molecular probes) and incubated for 10 min, washed, and air dried.
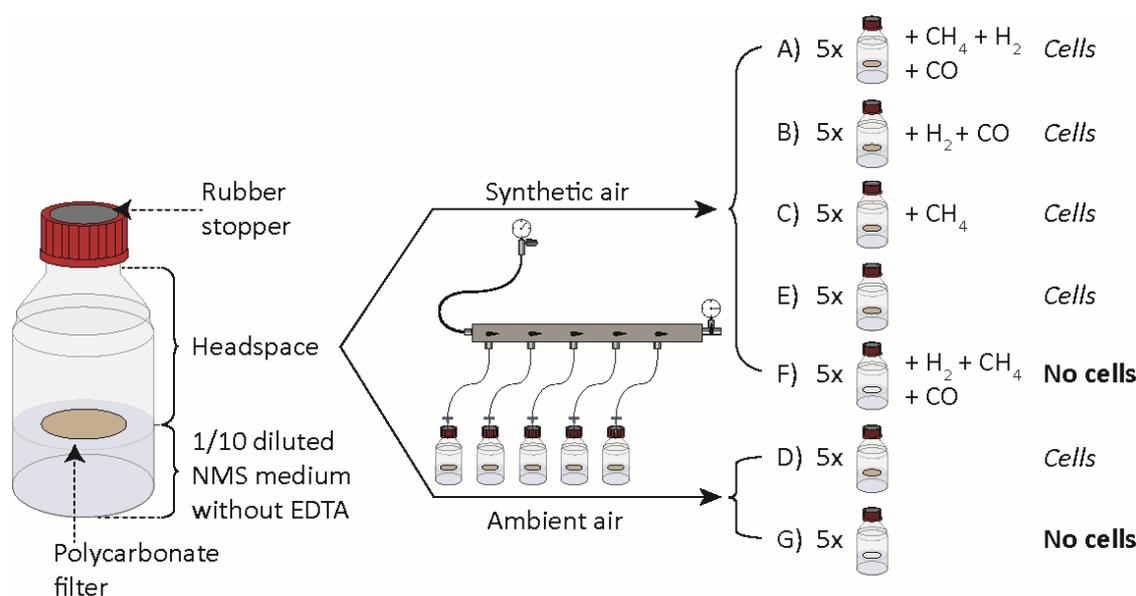
Figure 2. Experimental setup for experiment 1 to determine gas uptake by *M. gorgona* MG08. NMS: Nitrate mineral salts. Synthetic air containing less than 0.2 p.p.m.v. of CH_4, CO and H_2. Ambient air containing 1.84 p.p.m.v. CH_4, 0.4 p.p.m.v. CO and 0.7 p.p.m.v. H_2. All injections of CH_4, CO and H_2 resulted in a final concentration between 2 and 4 p.p.m.v of the respective gas.

In a 57-day experiment, we could show that *M. gorgona* MG08 is able to oxidize ambient air to sub-atmospheric concentrations of CH_4 and H_2 (Figure 3). In several of the incubations, CH_4 and H_2 concentrations reached below atmospheric levels already after 7 days, while CO concentrations did not decrease below ~0.45 p.p.m.v., which is close to the atmospheric levels in urban areas [16–19] and similar to the average ambient air concentrations measured in the laboratory (0.39 p.p.m.v.). Furthermore, the CH_4 oxidation rates did not depend on the CO and H_2 concentrations (Figure 3A,C,D), while CO and H_2 oxidation proceeded without the presence of CH_4 (Figure 3B).

The stabilization of the CO concentrations around 0.45 p.p.m.v. in bottles with *M. gorgona* MG08 cells, irrespective of the start concentrations, was unexpected (Figure 3A,B,D,E). In the cell-free controls, we observed increasing CO concentrations, reaching 2 p.p.m.v. during 57 days of incubation (Figure 3F,G), suggesting these observations to be due to an abiotic CO source. To identify the CO source, we measured CO, H_2 and CH_4 in several cell-free controls, including empty bottles (Figure 4). CO accumulation was detected in these liquid-free bottles, and even in headspaces exposed to only glass and rubber stoppers. Thus, we concluded that CO was released from the rubber stoppers, meaning that the stabilization of CO concentrations at ~0.45 p.p.m.v. in the presence of *M. gorgona* MG08 was a confirmation of continuous CO oxidation at near ambient air concentrations of ~0.45 p.p.m.v. The decreasing or increasing CH_4 and H_2 concentrations in abiotic controls (Figure 3F,G and Figure 4A–F), on the other hand, could be explained by leakage during long incubation times, as previously observed [27,28]. In addition to the fact that such long-term leakages have been observed previously, the rationale for this conclusion is that the concentrations inside the bottles always increased when the outside concentrations were higher, and decreased when the outside concentrations were lower.
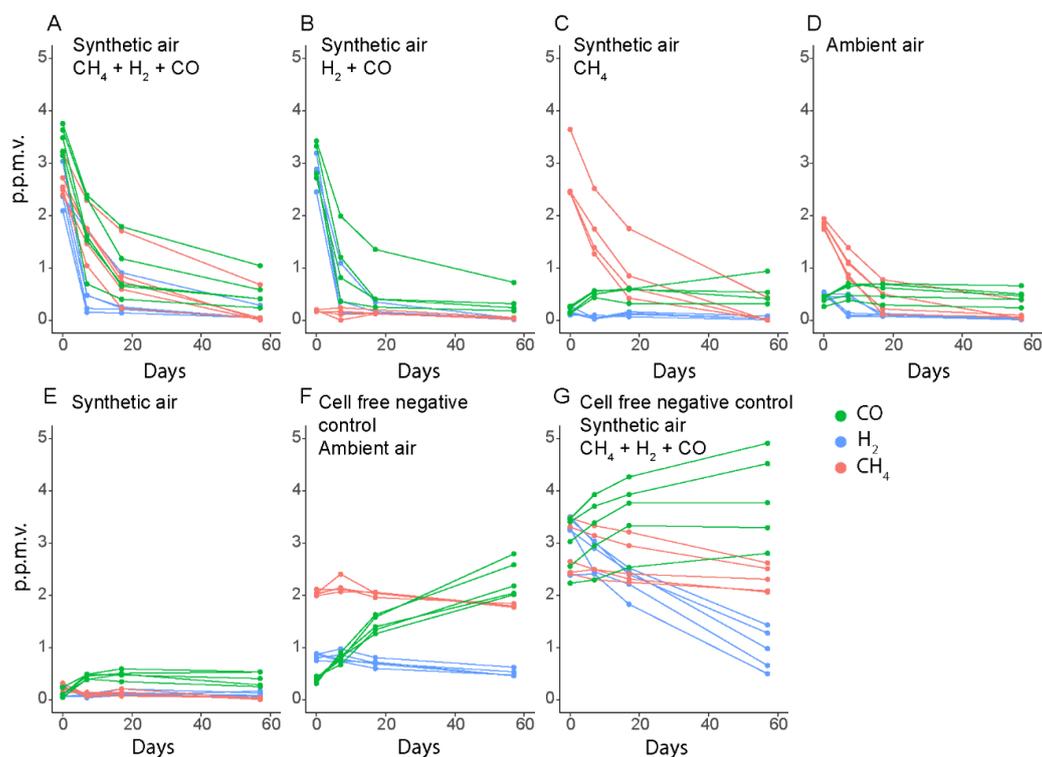
Figure 3. Oxidation of CH$_4$, CO, and H$_2$ by *M. gorgona* MG08. (**A**–**E**) contained polycarbonate filters with *M. gorgona* MG08 floating on nitrate mineral salts medium. (**F**,**G**) contained sterile filters. The lower H$_2$ concentrations at the start of the ambient air incubations with cells (**D**) compared to those without cells (**F**) are due to leaving prepared bottles overnight before measuring the first time point T$_0$, allowing some oxidation to already occur. All gases used were of the highest commercially available quality, 6.0 (99.9999% purity). The multiple data points in each color represent different biological replicates from the same condition. Synthetic air contained less than 0.2 p.p.m.v. of CH$_4$, CO and H$_2$.

Cell-Specific Oxidation Rates and Energy Yield

In order to estimate the CH$_4$, CO and H$_2$ uptake rates by *M. gorgona* MG08 at atmospheric concentrations, we tested and confirmed that the rate change of CH$_4$ uptake fulfilled the assumptions of first order rate kinetics (linear regression through the natural logarithm (LN) of rates over time $R^2 = 0.93$, $n = 15$). As microcolonies contained more than one layer of cells, filter cell counts were not possible. Instead, we performed DNA extractions and cell quantification with a DNA to cell count standard for a subset of the incubation bottles, showing that all but one floating filter contained between 38.1 and 68 million cells (Table S1). Based on these estimates, we calculated the cellular CH$_4$ oxidation rates at atmospheric CH$_4$ concentrations to 0.7–2.8 $\times$ 10^{-18} mol cell^{-1} h^{-1} at 20 °C ($n = 9$). Similarly, the H$_2$ and CO oxidation rates at atmospheric concentrations (average H$_2$ linear model $R^2 = 0.82$, $n = 10$, CO $R^2 = 0.68$, $n = 10$) were estimated to be 0.17–0.36 and 0.20–0.34 $\times$ 10^{-18} mol cell^{-1} h^{-1}, respectively ($n = 6$). These rates were corrected for the leakage and release of H$_2$ and CO, respectively. The CH$_4$ oxidation rates reflect the lower end of the atmospheric oxidation rates estimated for conventional MOB, which range from 0.2 to 17 $\times$ 10^{-18} mol cell^{-1} h^{-1} at 25 °C [6]. However, the rates of conventional MOB forming the basis for these estimates were measured at high CH$_4$ concentrations and thus do not necessarily represent the rates that would be obtained at atmospheric conditions.
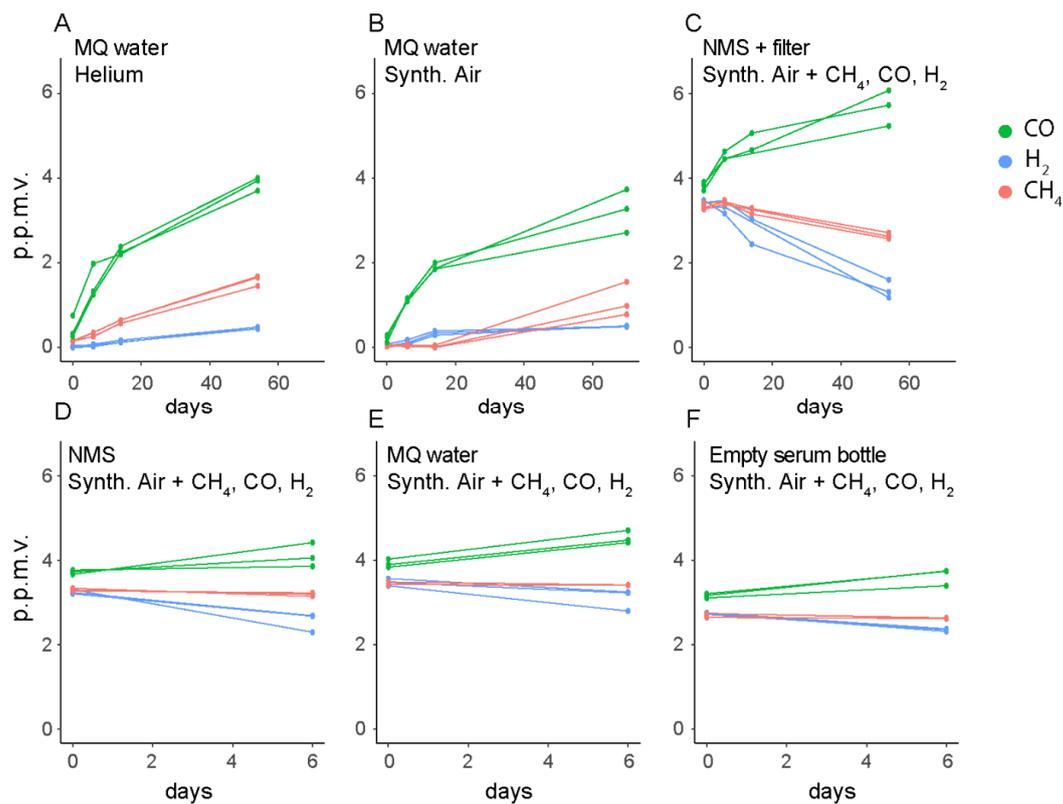
Figure 4. Gas leakage and other abiotic gas sources. (**A,B**) Long incubations of cell-free controls were performed to identify signs of CO and CH_4 leakage or release into or out of the bottles. (**C–F**) Short incubations of cell-free controls were considered sufficient to identify the same patterns of gas leakage and accumulation irrespective of bottle liquid and headspace composition. MQ: MilliQ. Synth. Air: Synthetic air. NMS: nitrate mineral salts. The multiple data points in each color represent different biological replicates from the same condition. Synthetic air contained less than 0.2 p.p.m.v. of CH_4, CO and H_2.

Based on the specific affinity estimated from oxidation rates at higher than atmospheric concentrations, the expected cell-specific CH_4 oxidation rate at atmospheric concentrations can be calculated [2]. From the oxidation rates measured at concentrations between 823 p.p.m.v. and 6% [4], we found that *M. gorgona* MG08 would have a cell specific CH_4 oxidation rate of 10×10^{-18} mol cell^{-1} h^{-1} when growing at atmospheric concentrations. However, these estimates are more than five times higher than the actual rate measurements at atmospheric concentrations provided in our current study. Thus, *M. gorgona* MG08 may not sustain the same catalytic properties or amount of particulate methane monooxygenase (pMMO) enzymes per cell during growth in air. At concentrations between 823 p.p.m.v. and 6%, we estimated the $K_{m(app)}$ of *M. gorgona* MG08 to be 4.905 [4], similar to that of various, presumed low affinity, MOB [2]. Although this indicated that *M. gorgona* MG08 has a low affinity for CH_4, the dependency of $K_{m(app)}$ on the V_{max} prevents us from determining the exact affinity unless we perform comparative kinetic experiments for purified enzymes from several MOB strains, or compare the CH_4 oxidation rates and pMMO concentrations of several different MOB. Thus, it seems plausible that life at atmospheric CH_4 concentrations can be sustained by low affinity enzymes, but it is still uncertain whether this is the case.

The energy yields per mol of CH_4, CO and H_2 under the provided experimental conditions (ambient air at 20 °C) were approximately −814, −522 and −472 kJ/mol, respectively, assuming the following reactions: [$CH_4 + 2O_2 \rightarrow 2H_2O + CO_2$], [$2CO + O_2 \rightarrow 2CO_2$], [$2H_2 + O_2 \rightarrow 2H_2O$]. Based on the measured rates, we find that *M. gorgona* MG08 is able to conserve approximately 0.47 kJ Cmol h^{-1} from the combined oxidation of CH_4, H_2 and CO

during growth in air at 20 °C (see data and full calculation in Table S1). This calculation implements our dry weight estimates for *M. gorgona* MG08 (Table S1). Interestingly, these estimates show that *M. gorgona* MG08 cells (length ~1.2 μm and width ~0.7 μm) are three times lighter (0.88×10^{-13} gDW cell^{-1}) than the lightest reported MOB [2]. The mass of a cell could affect its energy budget, as more energy might be needed to supply maintenance in a heavier cell, leaving less for growth. Additionally, a heavier cell might require more energy for growth. Thus, the low weight of *M. gorgona* MG08 may reflect a strategy to reduce energy costs for growth. In contrast to our findings, the previously reported soil CH_4 oxidation rates of 800×10^{-18} mol cell^{-1} h^{-1} [6] would provide 179 kJ Cmol h^{-1}. In order to examine this contradiction and test the validity of our data, we repeated parts of our experiment (condition with 5 p.p.m.v. of each gas CH_4, H_2 and CO) with shorter pre-incubation (4.5 months) to minimize growth stagnation and the possibility for the accumulation of dead cells on the floating filters.

Furthermore, we shortened the gas uptake experiment to less than one week of incubation to prevent gas leakage effects on rate estimates. This new setup provided highly precise estimates of CH_4, H_2 and CO uptake rates (Figure 5), which confirmed their first order nature (linear regression through LN of rates over time; CH_4 R^2 = 0.99, n = 5; H_2 R^2 = 0.99; CO R^2 = 0.99). Within this timeframe, the leakage of H_2 and CH_4 was negligible (Figure 5B,C), but the release of CO from the rubber stoppers occurred and was corrected for. Interestingly, similar but slightly lower cellular oxidation rates at atmospheric gas concentrations were estimated. The CH_4 uptake ranged from 0.32 to 1.3×10^{-18} mol cell^{-1} h^{-1}, the H_2 uptake from 0.63 to 2.1×10^{-18} mol cell^{-1} h^{-1}, and the CO uptake from 0.24 to 0.69×10^{-18} mol cell^{-1} h^{-1} at 20 °C. We suspect the differences were due to the higher accuracy of the uptake rates, and higher cell number estimates (average 9.54×10^7; Table S1), despite shorter incubation time (4.5 months). The number of initial cells that form colonies on the filters may vary between experiments, possibly due to small differences in the physiological state of the inoculum. This, and the possibility that colonies of a certain size reach a growth stagnation phase due to limiting concentrations of CH_4, H_2 and CO around the colony, can explain how additional pre-incubation time in the first experiment did not lead to higher cell numbers per filter than in the second experiment.
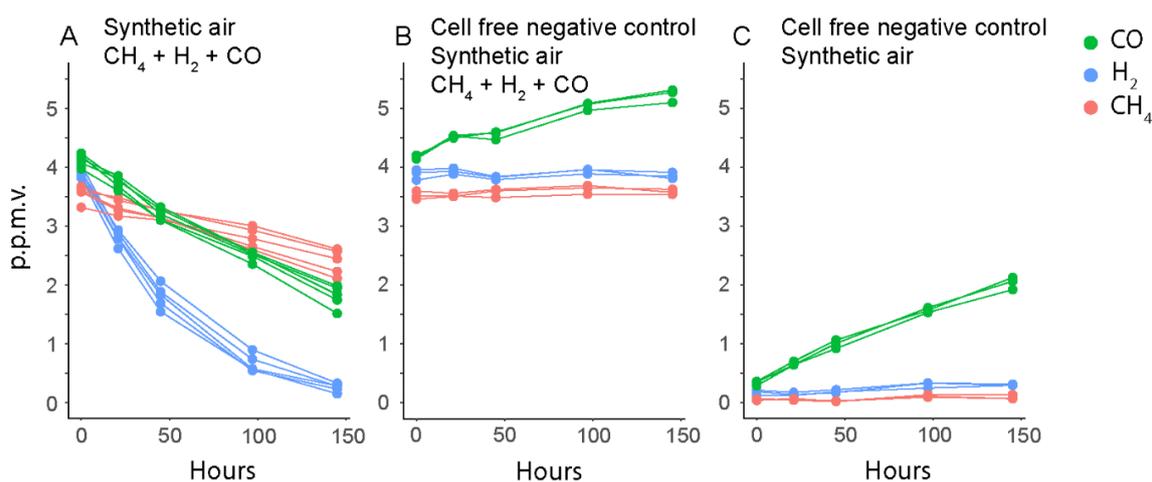
Figure 5. Oxidation of CH_4, CO, and H_2 by *M. gorgona* MG08. (**A**) filters with *M. gorgona* MG08 floating on nitrate mineral salts medium. (**B**,**C**) sterile filters floating on nitrate mineral salts medium. All gases used were of the highest commercially available quality, 6.0 (99.9999% purity). The multiple data points in each color represent different biological replicates from the same condition.

With these new numbers, we find that *M. gorgona* MG08 is able to conserve approximately 0.38 kJ Cmol h^{-1} (close to the 0.48 kJ Cmol h^{-1} estimated from the first experiment) during growth in air at 20 °C (Table S1). This is more than seven times lower

than the estimated average maintenance requirement of a bacterial population at 20 °C (2.8 kJ Cmol h^{-1}) [8]. However, this amount of energy is apparently sufficient to sustain the growth of *M. gorgona* MG08. Thus, we question whether the average maintenance requirement of bacteria [8] appropriately describes the constraints for growth on atmospheric trace gases by *M. gorgona* MG08 and other atmMOB.

With our numbers, the 2.8 kJ Cmol h^{-1} minimum requirement mentioned above could be achieved if approximately 14 million out of the ~100 million cells on a filter were active [8]. Alternatively, if cell density and thus weight (cell sizes do not seem to vary with CH$_4$ concentration) were lower during growth in air, an energy yield of 2.8 kJ Cmol h^{-1} or higher, could be achieved. The reason is that the same amount of energy would be distributed on less cellular mass than assumed for our empirical estimate of 0.38 kJ Cmol h^{-1}. Regardless, the rates of *M. gorgona* MG08 are substantially lower than those in high upland soils, where CH$_4$ oxidation rates of up of 800×10^{-18} mol cell^{-1} h^{-1}, almost 500 times faster than *M. gorgona* MG08, were measured. However, these were based on soil cell numbers estimated from DNA extractions and *pmoA* qPCR [6]. Thus, the numbers have possibly been underestimated, as DNA extractions from soils may provide less than 100% yields, qPCR quantification can be inhibited by soil-derived impurities in the DNA extract, and primer mismatches can result in an underrepresentation of copy numbers. Methanogenic archaea and acetogenic bacteria were recently found to require much less maintenance energy (0.2 kJ Cmol h^{-1}) than previously believed (9.8 kJ Cmol h^{-1}) [29]. According to the authors, the low maintenance energy was based on the low growth rates of these organisms, a feature that had not previously been taken into account. If true, low maintenance requirements at low growth rates could also explain how *M. gorgona* MG08 can sustain its slow growth at a limited energy budget.

We conclude that *M. gorgona* MG08 oxidizes the atmospheric trace gases CH$_4$, CO and H$_2$ to harvest energy for growth in air. The ability of *M. gorgona* MG08 to grow using air as its only source of energy and carbon relies not only on this metabolic flexibility, but also on its low energy requirements. Our findings suggest that a high CH$_4$ affinity is not a prerequisite to live on atmospheric CH$_4$.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/1/153/s1, Table S1: Supplementary data and calculations.

Author Contributions: A.T.T. conceived the study with input from T.S., M.M.S. and A.G.H. A.G.H. and M.M.S. established methods for filter cultivation and performed strain cultivation with input from A.T.T. T.S., M.L. and A.T.T. established methods for gas uptake experiments. T.S. performed gas uptake experiments with assistance from M.L. A.D. established and performed method for cell quantification. A.T.T. and T.S. analyzed the data. A.T.T. wrote the manuscript with input from all authors. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Van Amstel, A. Methane. A review. *J. Integr. Environ. Sci.* **2012**, *9*, 5–30. [CrossRef]
2. Knief, C.; Dunfield, P.F. Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. *Environ. Microbiol.* **2005**, *7*, 1307–1317. [CrossRef] [PubMed]
3. Dunfield, P.F. *The Soil Methane Sink. Greenhouse Gas. Sinks*; Reay, D.S., Hewitt, C.N., Smith, K.A., Grace, J., Eds.; CABI: Wallingford, UK, 2007; pp. 152–170.
4. Tveit, A.T.; Hestnes, A.G.; Robinson, S.L.; Schintlmeister, A.; Dedysh, S.N.; Jehmlich, N.; Von Bergen, M.; Herbold, C.; Wagner, M.; Richter, A.; et al. Widespread soil bacterium that oxidizes atmospheric methane. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 8515–8524. [CrossRef] [PubMed]
5. Knief, C. Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on pmoA as molecular marker. *Front. Microbiol.* **2015**, *6*. [CrossRef]

6. Kolb, S.; Knief, C.; Dunfield, P.F.; Conrad, R. Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. *Environ. Microbiol.* **2005**, *7*, 1150–1161. [CrossRef]
7. Bender, M.; Conrad, R. Kinetics of CH_4 oxidation in oxic soils exposed to ambient air or high CH_4 mixing ratios. *FEMS Microbiol. Lett.* **1992**, *101*, 261–270. [CrossRef]
8. Tijhuis, L.; Van Loosdrecht, M.C.M.; Heijnen, J.J. A thermodynamically based correlation for maintenance gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnol. Bioeng.* **1993**, *42*, 509–519. [CrossRef]
9. Cordero, P.R.F.; Bayly, K.; Leung, P.M.; Huang, C.; Islam, Z.F.; Schittenhelm, R.B.; King, G.M.; Greening, C. Atmospheric carbon monoxide oxidation is a widespread mechanism supporting microbial survival. *ISME J.* **2019**, *13*, 2868–2881. [CrossRef]
10. Ji, M.; Greening, C.; VanWonterghem, I.; Carere, C.R.; Bay, S.K.; Steen, J.A.; Montgomery, K.; Lines, T.; Beardall, J.; Van Dorst, J.; et al. Atmospheric trace gases support primary production in Antarctic desert surface soil. *Nature* **2017**, *552*, 400–403. [CrossRef]
11. Greening, C.; Carere, C.R.; Rushton-Green, R.; Harold, L.K.; Hards, K.; Taylor, M.C.; Morales, S.E.; Stott, M.B.; Cook, G.M. Persistence of the dominant soil phylum Acidobacteria by trace gas scavenging. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 10497. [CrossRef]
12. Greening, C.; Berney, M.; Hards, K.; Cook, G.M.; Conrad, R. A soil actinobacterium scavenges atmospheric H_2 using two membrane-associated, oxygen-dependent [NiFe] hydrogenases. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 4257. [CrossRef] [PubMed]
13. Greening, C.; Biswas, A.; Carere, C.R.; Jackson, C.J.; Taylor, M.C.; Stott, M.B.; Cook, G.M.; Morales, S.E. Genomic and metagenomic surveys of hydrogenase distribution indicate H_2 is a widely utilised energy source for microbial growth and survival. *ISME J.* **2016**, *10*, 761–777. [CrossRef] [PubMed]
14. Ray, A.E.; Zhang, E.; Terauds, A.; Ji, M.; Kong, W.; Ferrari, B.C. Soil Microbiomes With the Genetic Capacity for Atmospheric Chemosynthesis Are Widespread Across the Poles and Are Associated With Moisture, Carbon, and Nitrogen Limitation. *Front. Microbiol.* **2020**, *11*. [CrossRef] [PubMed]
15. World Meteorological Organization. The State of Greenhouse Gases in the Atmosphere Using Global Observations through 2018. *WMO GHG Bullet.* **2019**, *15*, 1–8. ISSN 2078-0796.
16. Khalil, M.A.K.; Rasmussen, R.A. The global cycle of carbon monoxide: Trends and mass balance. *Chemosphere* **1990**, *20*, 227–242. [CrossRef]
17. Novelli, P.C.; Masarie, K.A.; Lang, P.M. Distributions and recent changes of carbon monoxide in the lower troposphere. *J. Geophys. Res. Atmos.* **1998**, *103*, 19015–19033. [CrossRef]
18. Chi, X.; Winderlich, J.; Mayer, J.-C.; Panov, A.V.; Heimann, M.; Birmili, W.; Heintzenberg, J.; Cheng, Y.; Andreae, M.O. Long-term measurements of aerosol and carbon monoxide at the ZOTTO tall tower to characterize polluted and pristine air in the Siberian taiga. *Atmos. Chem. Phys.* **2013**, *13*, 12271–12298. [CrossRef]
19. Petrenko, V.V.; Martinerie, P.; Novelli, P.; Etheridge, D.M.; Levin, I.; Wang, Z.; Blunier, T.; Chappellaz, J.; KaiseriD, J.; Lang, P.; et al. A 60 yr record of atmospheric carbon monoxide reconstructed from Greenland firn air. *Atmos. Chem. Phys.* **2013**, *13*, 7567–7585. [CrossRef]
20. Novelli, P.C.; Lang, P.M.; Masarie, K.A.; Hurst, D.F.; Myers, R.; Elkins, J.W. Molecular hydrogen in the troposphere: Global distribution and budget. *J. Geophys. Res. Atmos.* **1999**, *104*, 30427–30444. [CrossRef]
21. Schmitz, R.A.; Pol, A.; Mohammadi, S.S.; Hogendoorn, C.; Van Gelder, A.H.; Jetten, M.S.M.; Daumann, L.J.; Camp, H.J.M.O.D. The thermoacidophilic methanotroph Methylacidiphilum fumariolicum SolV oxidizes subatmospheric H_2 with a high-affinity, membrane-associated [NiFe] hydrogenase. *ISME J.* **2020**, *14*, 1223–1232. [CrossRef]
22. Hakobyan, A.; Zhu, J.; Glatter, T.; Paczia, N.; Liesack, W. Hydrogen utilization by Methylocystis sp. strain SC2 expands the known metabolic versatility of type IIa methanotrophs. *Metab. Eng.* **2020**, *61*, 181–196. [CrossRef] [PubMed]
23. R Development Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2013. Available online: http://www.R-project.org/ (accessed on 23 January 2020).
24. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016. Available online: https://ggplot2.tidyverse.org (accessed on 23 January 2020).
25. Pieja, A.J.; Sundstrom, E.R.; Criddle, C.S. Poly-3-Hydroxybutyrate Metabolism in the Type II Methanotroph Methylocystis parvus OBBP. *Appl. Environ. Microbiol.* **2011**, *77*, 6012–6019. [CrossRef] [PubMed]
26. Ratcliff, W.C.; Kadam, S.V.; Denison, R.F. Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiol. Ecol.* **2008**, *65*, 391–399. [CrossRef]
27. Lange, S.F.; Allaire, S.E.; van Bochove, E. Transfer of CO_2, N_2O and CH_4 to butyl rubber (polyisobutylene) septa during storage. *J. Environ. Monit.* **2008**, *10*, 775–777. [CrossRef] [PubMed]
28. Magnusson, T. A method for equilibration chamber sampling and gas chromatographic analysis of the soil atmosphere. *Plant Soil* **1989**, *120*, 39–47. [CrossRef]
29. Bonk, F.; Popp, D.; Weinrich, S.; Sträuber, H.; Becker, D.; Kleinsteuber, S.; Harms, H.; Centler, F. Determination of Microbial Maintenance in Acetogenesis and Methanogenesis by Experimental and Modeling Techniques. *Front. Microbiol.* **2019**, *10*. [CrossRef]

Paper 2

1　Physiological Basis for Atmospheric Methane Oxidation and

2　Methanotrophic Growth on Air

3　Tilman Schmider[a*], Anne Grethe Hestnes[a], Julia Brzykcy[b], Hannes Schmidt[c], Arno

4　Schintlmeister[d], Benjamin R. K. Roller[d], Ezequiel Jesús Teran[e,f], Andrea Söllinger[a], Oliver

5　Schmidt[a], Martin F. Polz[d], Andreas Richter[c], Mette M. Svenning[a], Alexander T. Tveit[a*]

6　[a]Department of Arctic and Marine Biology, Faculty of Biosciences, Fisheries and Economics,

7　UiT - The Arctic University of Norway, 9037 Tromsø, Norway;

8　[b]Department of Geomicrobiology, Institute of Microbiology, Faculty of Biology, University of

9　Warsaw, 02-096 Warsaw, Poland

10　[c]Department of Microbiology and Environmental Systems Science, Division of Terrestrial

11　Ecosystem Research, University of Vienna, 1030 Vienna, Austria;

12　[d]Department of Microbiology and Environmental Systems Science, Division of Microbial

13　Ecology, University of Vienna, 1030 Vienna, Austria;

14　[e]Centro de Investigaciones en Física e Ingeniería del Centro de la Provincia de Buenos Aires

15　(CIFICEN-UNCPBA-CONICET-CICPBA), Pinto 399, Tandil (7000), Argentina

16　[f]Universidad Nacional del Centro de la Provincia de Buenos Aires, Facultad de Ciencias Exactas,

17　Instituto de Física Arroyo Seco (IFAS), Pinto 399, Tandil (7000), Argentina

18　*Corresponding author. Email: tilman.schmider@uit.no; alexander.t.tveit@uit.no

19

20   Abstract

21   Atmospheric methane oxidizing bacteria (atmMOB) constitute the sole biological sink for

22   atmospheric methane and have been discovered worldwide over the past decades. Still, the

23   physiological basis allowing atmMOB to grow "on air" is not understood. Here we assess the

24   ability and strategies of seven methanotrophic species to grow with air as sole energy, carbon,

25   and nitrogen source. Four species, including three outside the canonical atmMOB group USCα,

26   enduringly oxidized atmospheric methane, carbon monoxide, and hydrogen during 12 months

27   of growth on air. These four species exhibited distinct substrate preferences implying the

28   existence of multiple metabolic strategies to grow on air. Despite simultaneous oxidation of

29   atmospheric methane, carbon monoxide, and hydrogen, the estimated energy yields of the

30   atmMOB were substantially lower than previously assumed necessary for cellular

31   maintenance in atmMOB and other aerobic microorganisms. Moreover, the atmMOB also

32   covered their nitrogen requirements from air. During growth on air, the atmMOB allocated

33   their proteome by decreasing investments in biosynthesis while increasing investments in

34   trace gas oxidation. Additionally, we demonstrate high apparent affinities for methane by

35   atmMOB in pure culture, similar to the affinities observed in upland soils more than two

36   decades ago. Nevertheless, we show that the apparent specific affinity, rather than the

37   apparent affinity, is the appropriate model to assess the efficiency of atmospheric methane

38   utilization. Our work shows that atmMOB grow on the trace concentrations of methane,

39   carbon monoxide, and hydrogen present in air and outlines the metabolic strategies that

40   enable atmMOB to mitigate important greenhouse gases.

41

42

Introduction

During the first two decades after emission to the atmosphere, methane (CH_4) is a greenhouse gas 80 times more potent than carbon dioxide (CO_2)[1,2]. Since 2007, the atmospheric CH_4 concentration (1905 p.p.b.v. in July 2022 https://gml.noaa.gov/ccgg/trends_ch4/), that is responsible for approximately 20% of the direct radiative forcing, has been increasing rapidly[1]. The CH_4 increase further accelerated in 2014 and is linked to several causes: A decline in the atmospheric concentration of hydroxyl radicals (OH) which is the main sink of atmospheric CH_4, as OH oxidize CH_4 in the atmosphere[3–5]; anthropogenic emissions from fossil fuel, agricultural, and waste sources[6]; increased microbial CH_4 production in wetlands which suggests that current increases are also driven by feedback responses to global warming[7]. Atmospheric CH_4 oxidizing bacteria (atmMOB), a subgroup of aerobic methanotrophs, that oxidize CH_4 at its atmospheric trace concentration are the only known biological sink of atmospheric CH_4. Compared to the OH sink (~500 Tg), the biological sink is rather small as it removes approximately 30 Tg (11 – 49 Tg) CH_4 from the atmosphere every year[4]. However, the biological sink has the potential to grow with increasing CH_4 concentrations. This is of particular importance as the decline of atmospheric OH, caused by reaction with atmospheric hydrogen (H_2) and other gases, might accelerate due to increasing H_2 emissions from a hydrogen-based economy[8,9]. Additionally, the biological sink is within reach of management practices devised to maximize its natural potential and harness it for CH_4 removal[10,11]. Yet, substantial uncertainties concerning the size of the biological sink, and the ecology and metabolic basis for growth on atmospheric CH_4 by atmMOB, caused by a historical lack of atmMOB in pure culture, has impeded our ability to study, manage, and exploit the sink[10]. In

65   this study, by screening seven methanotrophic species, we have outlined the physiological

66   basis that enables atmMOB to grow on atmospheric CH_4 and serve as an atmospheric CH_4 sink.

67   In 1992, ten years after Harriss *et al.* reported the first indications of atmospheric CH_4

68   oxidation by microorganisms[12], Bender and Conrad concluded from biphasic CH_4 oxidation

69   kinetics of soils that an unknown group of methanotrophs might be responsible for

70   atmospheric CH_4 oxidation[13]. Since then, two major questions have remained partially

71   unanswered: Which methanotrophs are responsible for oxidation of atmospheric CH_4? How

72   can these organisms survive and grow despite the apparent energetic limitations inherent to

73   the oxidation of the low atmospheric CH_4 concentrations?

74   Isotopic labeling studies revealed that members of *Alpha-* and *Gammaproteobacteria*

75   contributed to atmospheric CH_4 oxidation and assigned them to the upland soil clusters alpha

76   and gamma (USCα and USCγ)[14–16]. Several environmental and ecological studies have ascribed

77   atmospheric CH_4 oxidation mainly to these two clusters[17–20]. However, over the years, studies

78   targeting methanotrophs in upland soils have reported the presence of alphaproteobacterial

79   methanotrophs outside the USCα[14,21–24]. These observations suggest that also "conventional"

80   methanotrophs (methanotrophs assumed to grow only at high CH_4 concentrations), from

81   genera like *Methylocapsa*, *Methylosinus* and *Methylocystis*, could contribute to the

82   atmospheric CH_4 sink.

83   Dunfield[25] summarized three potential lifestyles of atmMOB that might enable cellular

84   maintenance and growth at the low CH_4 concentrations in air and the associated energy

85   limitation: (i) Flush feeding on high CH_4 concentrations generated periodically in deeper soil

86   layers, in addition to atmospheric CH_4 oxidation; (ii) An oligotrophic lifestyle based on

87 atmospheric CH_4 as sole carbon and energy source; (iii) A mixotrophic lifestyle to utilize other

88 substrates for energy conservation in addition to CH_4.

89 Flush feeding (i) is supported by the declining potential of methanotrophs to oxidize

90 atmospheric CH_4 after several months of CH_4 starvation[25,26]. A study on conventional

91 methanotrophs in rice paddy soils showed that methanotrophs regained the ability to oxidize

92 atmospheric CH_4 after exposure to high CH_4 concentrations[27]. A high specific affinity (a_A^0) for

93 CH_4 has been suggested as the key trait of oligotrophic atmMOB (ii) to enable growth with air

94 as their only energy and carbon source[25,28]. This assumes that cellular energy requirements

95 for maintenance are 4.5 kJ per carbon mole of biomass per hour (C-mol^{-1} h^{-1}) (at 25°C)[29]. Thus,

96 to survive, oligotrophic atmMOB presumably need an atmospheric CH_4 oxidation rate high

97 enough to meet these energy requirements. Such a rate can be achieved by the combination

98 of a high affinity for CH_4, reflected in a low half saturation constant (K_m), and a high maximum

99 CH_4 oxidation rate (V_{max}), the fraction of V_{max} and K_m being referred to as a_A^0 [30]. This theory is

100 supported by low K_m values for CH_4 found in several soils[13]. However, in a later study the cell

101 specific CH_4 oxidation of USCα members was estimated to be 2.9 to 40 times lower than the

102 presumed rate needed for cellular maintenance[31]. Therefore, the authors considered that a

103 mixotrophic lifestyle (iii) could be the basis for atmospheric CH_4 oxidation. In line with this, a

104 recent study reported the simultaneous oxidation of atmospheric H_2, carbon monoxide (CO),

105 and CH_4 by *Methylocapsa gorgona* MG08, the first known methanotroph and USCα member

106 in pure culture that can grow "on air" (with air as sole energy and carbon source)[32,33]. Despite

107 its mixotrophic lifestyle, *M. gorgona* MG08 did not conserve enough energy (0.38 kJ C-mol^{-1}

108 h^{-1}) to cover the 2.8 kJ C-mol^{-1} h^{-1} theoretically required to support maintenance at 20°C (2.8

109 kJ C-mol^{-1} h^{-1} at 20°C correspond to 4.5 kJ C-mol^{-1} h^{-1} at 25°C)[29,32], questioning whether this

110 maintenance energy value and thus a high a_A^0 is a relevant benchmark for the physiological

5

capabilities of trace gas oxidizing bacteria. While the initial isolation and study of *M. gorgona*

MG08 led to important advancements in our understanding of atmMOB, our knowledge of

the metabolic basis allowing atmMOB to grow on atmospheric CH_4 remained limited. Recent

energy estimates relied on the untested assumptions that all cells remained active over time

and could not grow on air without CH_4, H_2 and CO. Furthermore, these methodologically

limited of estimates had only been carried out on one strain, *M. gorgona* MG08, and had not

been combined with other methods to reveal the metabolic strategies associated to the

energy yields. On this background and to test if methanotrophs outside the USCα and USCγ

can grow by oxidizing atmospheric CH_4, H_2 and CO, we used filter cultivation to screen six

alphaproteobacterial, methanotrophic strains and one gammaproteobacterial,

methanotrophic strain for their ability to grow on air. We selected the six alphaproteobacterial

strains *Methylocapsa gorgona* MG08, *Methylosinus trichosporium* OB3b, *Methylocystis rosea*

SV97, *Methylocapsa aurea* KYG, *Methylocapsa acidiphil*a B2, and *Methylocapsa palsarum*

NE2[33–38] since members of *Methylocapsa*, *Methylosinus* and *Methylocystis* have been

observed in net sinks for atmospheric CH_4 (upland soils). As representative methanotroph

from the *Gammaproteobacteria* we included *Methylobacter tundripaludum* SV96. To outline

the physiological basis for growth on air, we performed trace gas oxidation experiments,

estimated energy yields from the oxidation of trace gases in air, used comparative proteomics

to investigate proteome allocation, and determined the a_A^0 for CH_4 during growth on air. Based

on the lack of knowledge about nitrogen limitation of atmMOB[39], we also evaluated their

ability to grow on the nitrogen sources available in air.

Results and Discussion

Colony formation, trace gas oxidation, and cellular energy yield during growth on air

To test the ability of the seven selected methanotrophs to grow on air, we incubated each strain on filters floating on carbon free medium with ambient air as the sole carbon and energy source, hereafter referred to as filter culture. Microscopy demonstrated that all strains except *Methylobacter tundripaludum* SV96, the gammaproteobacterial strain, formed colonies during six months of incubation (Fig. 1A). To test the metabolic activity of the six months old strains, we performed trace gas oxidation experiments (Fig. 1A, 1B). During these experiments, filter cultures were floating on mineral medium in bottles with trace concentrations of CH_4, H_2, and CO in the headspace. The strains, *M. aurea* KYG, *M. gorgona* MG08, *M. palsarum* NE2, and *M. rosea* SV97, hereafter referred to as atmMOB, oxidized CH_4, CO, and H_2, or CH_4 and CO to sub-atmospheric concentrations showing strain-specific oxidation patterns (Fig. 1A, Supplementary Fig. 1). *M. aurea* KYG oxidized CO at the highest rate, followed by CH_4 and H_2. *M. gorgona* MG08 oxidized H_2 at the highest rate, followed by CO and CH_4. *M. rosea* SV97 oxidized CO at the highest rate, followed by CH_4 and H_2. *M. palsarum* NE2 oxidized CH_4 and CO at similar rates but did not oxidize H_2. The gas oxidation patterns were similar after 12 months of incubation with air (Supplementary Fig. 1). To verify growth on the three trace gases in air, we incubated *M. gorgona* MG08 cells on filters under two different atmospheres: One of synthetic air without the trace gases CH_4, CO, and H_2 (Gas composition: 400 p.p.m.v. CO_2 and 20.9% O_2 in N_2) and another of ambient air. Growth by colony formation was only observed in the ambient air control, whereas no growth was observed in synthetic air (Supplementary Fig. 2). This and the repeated observations of trace gas oxidation confirm that these strains can live and grow with air as the sole energy and carbon source. The observed oxidation of at least one atmospheric trace gas in addition to

156 CH_4 demonstrates that all four strains are mixotrophic, matching the proposition by Dunfield

157 that mixotrophy could be a physiological basis for atmMOB[25]. Additionally, the strain-specific

158 trace gas uptake patterns demonstrate substantial metabolic differences between these four

159 strains, indicating that multiple metabolic strategies can support oxidation of atmospheric CH_4

160 for growth. These results also demonstrated that the enduring oxidation of atmospheric CH_4

161 after 12 months of growth on air is not restricted to members of the USCα and USCγ, but also

162 include members from the genera *Methylocystis* and *Methylocapsa*: *M. aurea* KYG, *M.*

163 *palsarum* NE2, and *M. rosea* SV97 (Fig. 2).

164

Despite formation of colonies when exposed to air only, *M. acidiphila* B2 and *M. trichosporium* OB3b did not oxidize trace gases after six months of incubation (Fig. 1A). The ability of these two strains to produce polyhydroxyalkanoates (PHA) as storage compounds[36,40] might serve as an explanation for the initial colony formation. Possibly, the two strains accumulated PHA during pre-cultivation at 20% CH_4, and then sustained their growth on air by utilizing PHA and atmospheric CH_4 as energy and carbon sources until the depletion of their storage compounds. A study showing that both strains could not stay active at low CH_4 concentrations[21] supports our observations, but further studies are needed to clarify whether the initial colony formation is based on storage compounds.
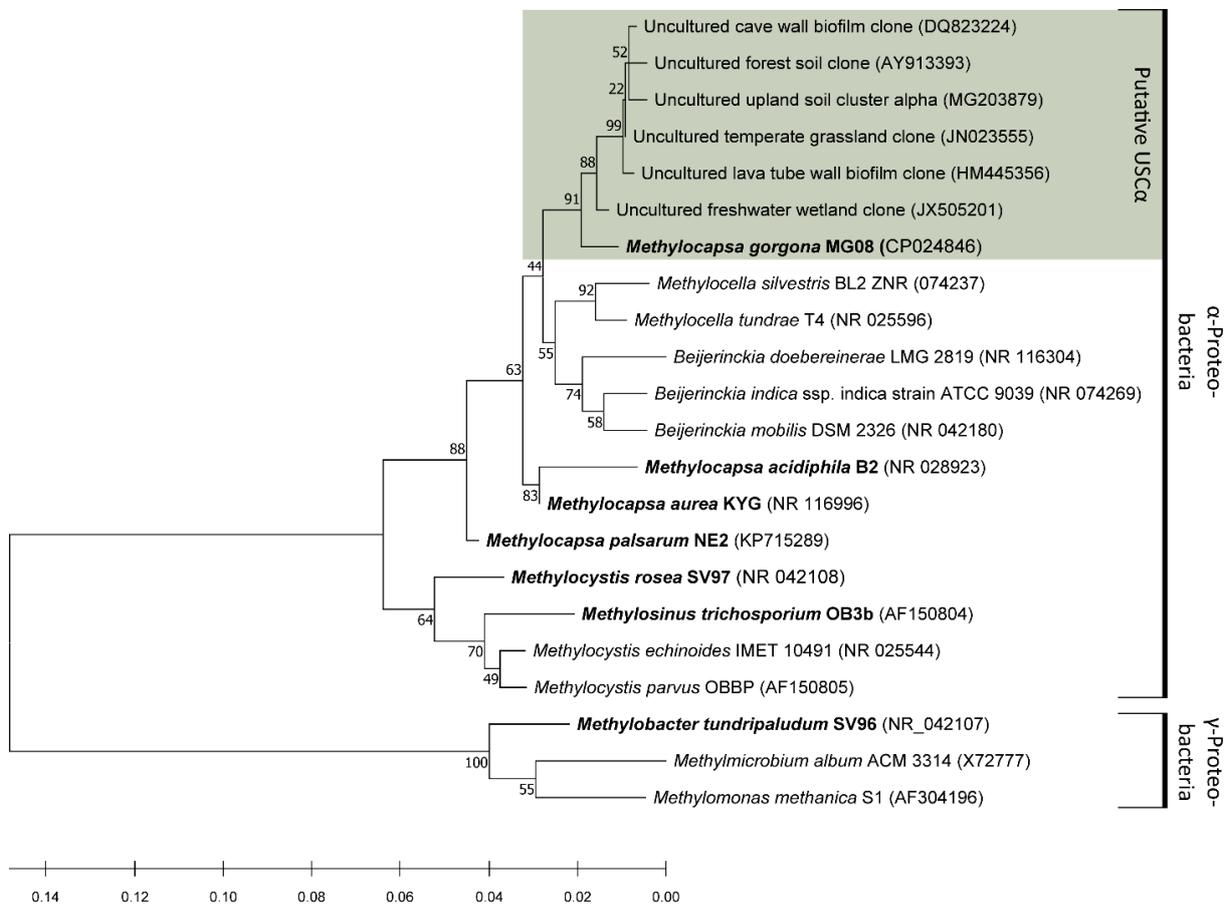
Figure 2. Unrooted maximum-likelihood tree[41,42]. Full length 16S rRNA gene based phylogenetic relationship of the respective methanotrophs and the USCα. The species investigated in this study are indicated in bold print. The percentage of trees in which the associated taxa clustered together is shown next to the branches. NCBI accession numbers are given in brackets.

184

185 Next, we asked whether atmMOB can obtain enough energy from growth on air to meet the

186 basic maintenance energy of 2.8 kJ C-mol^{-1} h^{-1} at 20°C postulated previously[28]. To calculate

187 the strain-specific energy yields in C-mol, we first estimated the cellular energy yields based

188 on the measured CH_4, H_2, and CO oxidation rates. *M. aurea* KYG yielded 6.89 x 10^{-12} J cell^{-1} h^{-1}

189 (n = 5, SD = 3.22 x 10^{-12}), *M. gorgona* MG08 2.21 x 10^{-12} J cell^{-1} h^{-1} (n = 5, SD = 4.36 x 10^{-13}), *M.*

190 *palsarum* NE2 3.09 x 10^{-12} J cell^{-1} h^{-1} (n = 5, SD = 2.90 x 10^{-13}), and *M. rosea* SV97 3.879 x 10^{-12}

191 J cell^{-1} h^{-1} (n = 5, SD = 1.57 x 10^{-12}) by the oxidation of either two or three trace gases in air

192 (Fig. 1B). Due to the higher free energy potential and atmospheric concentration of CH_4

193 compared to atmospheric H_2 and CO, the energy estimates predict CH_4 as the major energy

194 source for all four strains. Our estimates derive from the Gibbs free energy change of the

195 following reactions at atmospheric conditions: $CH_4 + 2O_2 \rightarrow 2H_2O + CO_2$, $2H_2 + O_2 \rightarrow$

196 $2H_2O$, and $2CO + O_2 \rightarrow 2CO_2$ that amount to -797.4 kJ mol^{-1}, -236.8 kJ mol^{-1}, and -199.9 kJ

197 mol^{-1}, respectively. However, these numbers do not account for the energy required for

198 activation of CH_4 by the pMMO or production cost of the enzymes involved in energy

199 conservation from the gases. While the oxidation of H_2 and CO is catalyzed by one enzyme[43],

200 the oxidation of CH_4 to CO_2 by atmMOB involves at least seven enzymes (Fig. 4). Thus, due to

201 the larger investments required for energy conservation from CH_4, the gases CO and H_2 might

202 play more important roles as energy sources for growth on trace gases in air than indicated

203 by the energy calculations alone. Considering the cellular dry masses and carbon content

204 (Supplementary table S3) of *M. gorgona* MG08, *M. aurea* KYG, *M. palsarum* NE2, and *M. rosea*

205 SV97, the energy yields per cell and hour translates to 0.40 kJ C-mol^{-1} h^{-1} (SD = 0.08 kJ C-mol^{-1}

206   h^{-1}), 0.71 kJ C-mol^{-1} h^{-1} (SD = 0.33 kJ C-mol^{-1} h^{-1}), 0.38 kJ C-mol^{-1} h^{-1} (SD =0.04 kJ C-mol^{-1} h^{-1}),

207   and 0.65 kJ C-mol^{-1} h^{-1} (SD = 0.26 kJ C-mol^{-1} h^{-1}), at 20°C, respectively (Fig. 3). These estimated

208   energy yields for *M. gorgona* MG08, *M. aurea* KYG, *M. palsarum* NE2, and *M. rosea* SV97 are

209   3.9 – 7.4 times lower than the reported average energy requirements for cellular maintenance

210   in aerobic bacteria (2.8 kJ C-mol^{-1} h^{-1} at 20°C correspond to 4.5 kJ C-mol^{-1} h^{-1} at 25°C)[29,32].

211   The energy estimation assumes that all cells of the filter cultures actively contributed to the

212   observed oxidation rate. Thus, in case of inactive cells, the cellular oxidation rates and the

213   energy yields might have been underestimated. However, while the applied cell quantification

214   (see "Methods") considers only intact cells without assessing cellular activity, Nanoscale

215   secondary ion mass spectrometry (NanoSIMS) based ^{15}N incorporation confirmed activity of

216   all measured cells (see section below: "Growth on nitrogen from air"), supporting our

217   approach for energy yield estimations.

218   Thus, our observations contradict the basic energy premise for atmospheric CH$_4$ oxidizing

219   bacteria[28]. Our energy estimations correspond to the similarly low energy estimates

220   previously reported for *M. gorgona* MG08[32] and that of acetogenic and methanogenic

221   microorganisms (0.2 kJ C-mol^{-1} h^{-1} at 37°C)[44].

222
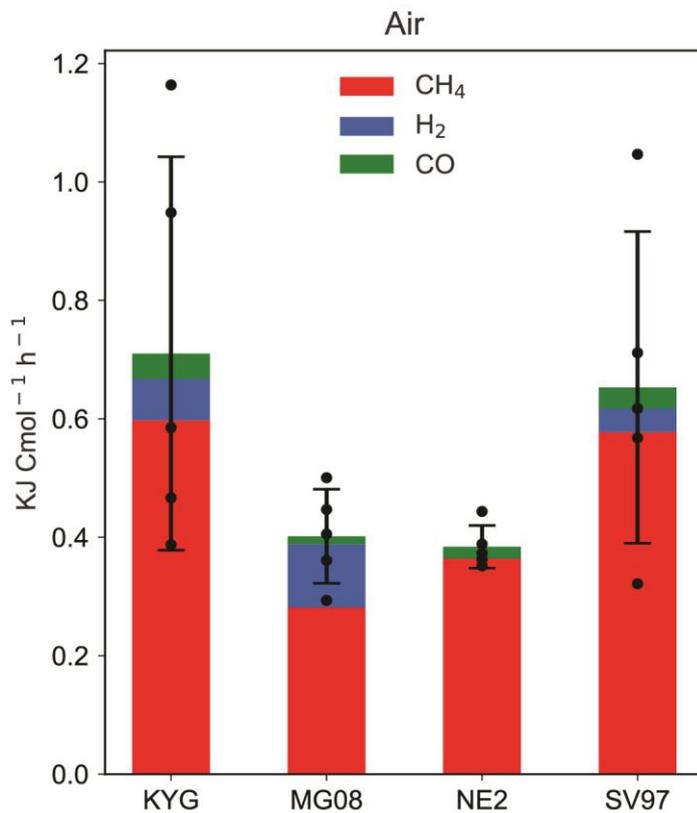
Figure 3. Total energy yield of *M. aurea* KYG, *M. gorgona* MG08, *M. palsarum* NE2, *M. rosea* SV97 from the oxidation of atmospheric CH_4, H_2, and CO in kJ per mol cellular carbon (C-mol) and hour (Supplementary table S3). Colors indicate the contribution of the individual trace gases to the total energy yield. Error bars represent the standard deviation (n = 5). Black dots indicate the total energy yield from H_2, CO, and CH_4 of the respective replicates.

Comparative proteomics

We further investigated the cellular adjustments required for life on air by *M. gorgona* MG08,

M. rosea SV97, and *M. palsarum* NE2 by comparing the proteomes of the three strains when

exposed to air (~1.9 p.p.m.v. CH_4) and when exposed to high CH_4 concentrations (~1000

p.p.m.v. CH_4) in air. Correspondence analyses (CA) of relative protein abundances at the two

CH_4 concentrations revealed a clear difference in proteome allocation. This is shown by the

separation of samples from the two conditions along the first CA dimension, which accounted

for 79.3%, 76.5%, and 69.6% of the total inertia for *M. gorgona* MG08, *palsarum* NE2, and *M.*

rosea SV97, respectively (Supplementary Fig. 3). Thus, the CA indicate that the largest shifts

236 in the proteomes occurred as responses to changes in CH_4 concentration. To identify which

237 proteins contributed most to these shifts, we identified the proteins (top 10 %) with the

238 largest contribution to the first-dimension inertias of the CA and plotted their abundances

239 across the two conditions (Supplementary Fig. 4). We found major protein expression shifts

240 within a variety of functional categories (based on hierarchical EggNOG[45] annotations), but for

241 all three strains, a large proportion was related to core metabolisms, including transcription,

242 translation, growth, energy metabolism, and amino acid and carbohydrate transport. A

243 prominent trend observed for all three strains was that proteins associated with the

244 categories "Cell cycle control, cell division, chromosome partitioning" and "Cell

245 wall/membrane/envelope biogenesis" shifted towards lower relative abundances, hereafter

246 referred to as "downregulation", at atmospheric CH_4 compared to the 1000 p.p.m.v. CH_4

247 treatment. This pattern suggests that atmMOB lower the allocation of resources for growth

248 when CH_4 availability is low, which is in line with previously reported differences in colony

249 growth over time when comparing incubations on atmospheric and 1000 p.p.m.v. CH_4

250 concentrations[28]. We also observed major shifts in protein abundances for the categories

251 "Energy production and conversion" and "Carbohydrate transport and metabolism", including

252 proteins involved in trace gas oxidation and carbon assimilation (Supplementary tables S12-

253 14). Based on that and the differences in trace gas oxidation patterns between the strains (Fig.

254 1), the relative abundances of proteins involved in trace gas oxidation, carbon assimilation via

255 the serine cycle (Fig. 4), and the electron transport chain (Supplementary Fig. 5) were further

256 investigated.

257
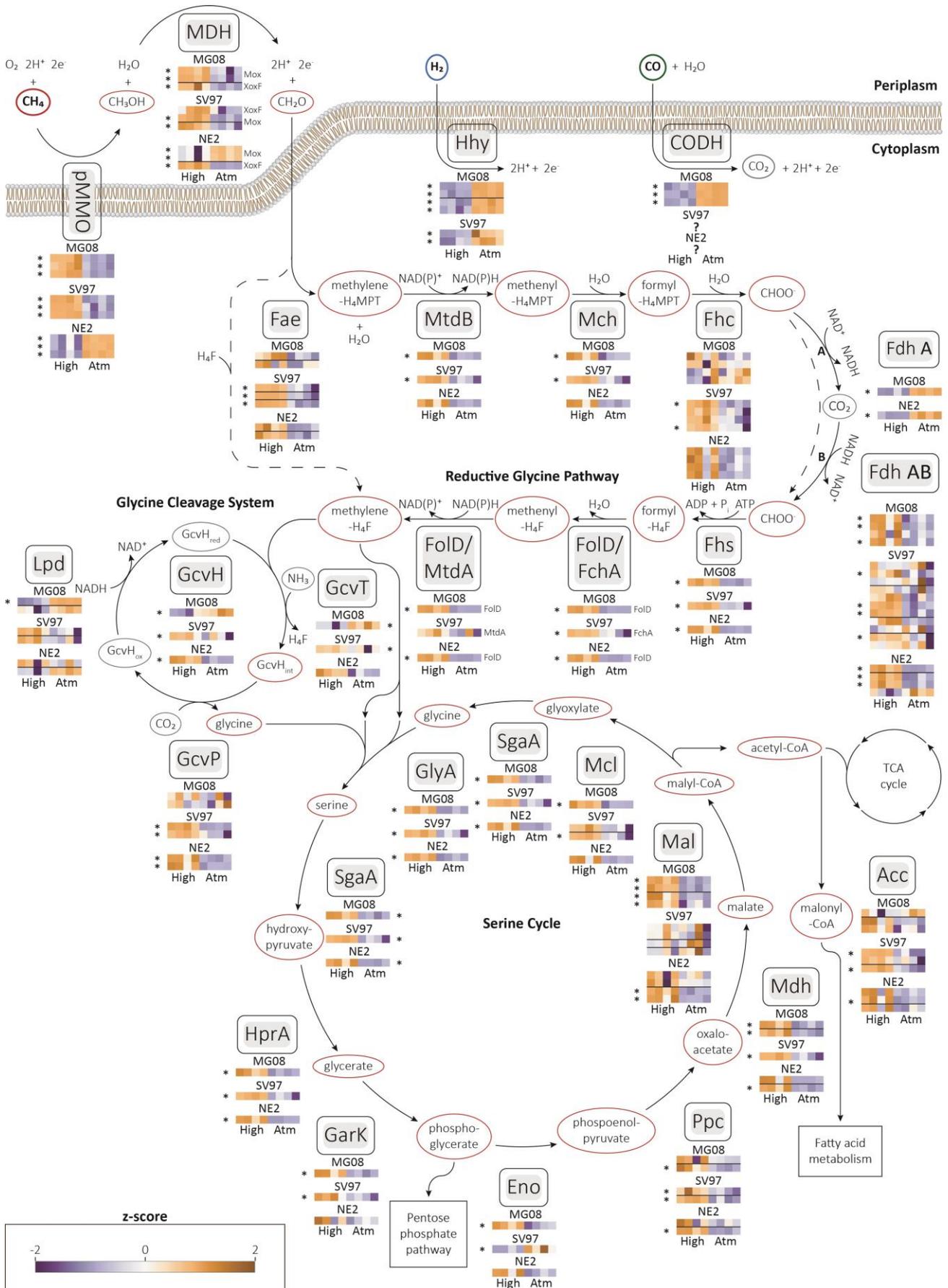
260 Figure 4. Comparative proteomics of *M. gorgona* MG08, *M. rosea* SV97, and *M. palsarum* NE2 exposed to 1000 p.p.m.v. CH$_4$
261 (High) in air and 1.9 p.p.m.v. (Atm) CH$_4$ in air (Supplementary table S5). Normalized and standardized expression of enzymes
262 involved in the central carbon and energy metabolism is shown. High relative abundance = orange, low relative abundance
263 expression = blue. * indicates significant difference in expression between treatments (two-sided t-test). Horizontal lines in
264 the heatmaps separate operons of enzymes catalyzing the same reaction. ? = unknown enzyme. Abbreviations: pMMO –
265 particulate methane monooxygenase, MDH – methanol dehydrogenase (Mox) and putative lanthanide-dependent methanol
266 dehydrogenase (XoxF), Hhy – [NiFe] group 1h hydrogenase, CODH – [MuCO] class I carbon monoxide dehydrogenase, Fae –
267 formaldehyde activating enzyme, MtdB – NAD(P)-dependent methylene-tetrahydromethanopterin dehydrogenase, Mch –
268 methenyl-tetrahydromethanopterin cyclohydrolase, Fhc – formyltransferase/hydrolase, Fdh **A** – NAD-dependent formate-
269 dehydrogenase, Fdh **AB** – molybdopterin binding reversible formate dehydrogenase/CO$_2$ reductase, Fhs – formate-
270 tetrahydrofolate ligase, FolD – bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/ 5,10-methenyl-
271 tetrahydrofolate cyclohydrolase, FchA – methenyltetrahydrofolate cyclohydrolase, MtdA – NADP-dependent
272 methylenetetrahydrofolate dehydrogenase, GcvH – glycine cleavage system H protein, GcvT – aminomethyltransferase, GcvP
273 - glycine dehydrogenase, Lpd – dihydrolipoyl dehydrogenase, GlyA – serine hydroxymethyltransferase, SgaA – Serine-
274 glyoxylate aminotransferase, HprA – glycerate dehydrogenase, GarK – 2-glycerate kinase, Eno – enolase, Ppc –
275 phosphoenolpyruvate carboxylase, Mdh – malate dehydrogenase, Mal – malate-CoA ligase, Mcl – L-malyl-CoA lyase, Acc –
276 Acetyl-CoA carboxylase, NAD$^+$ – nicotinamide adenine dinucleotide, NADP$^+$ – nicotinamide adenine dinucleotide phosphate,
277 ATP – adenosine triphosphate.

278 All three strains expressed a particulate methane monooxygenase (pMMO) that catalyzes the

279 hydroxylation of CH$_4$ to methanol (CH$_3$OH). *M. gorgona* MG08 and *M. rosea* SV97 contained

280 higher relative abundances, hereafter referred to as "upregulation", of pMMO at 1000

281 p.p.m.v. CH$_4$, whereas *M. palsarum* NE2 upregulated pMMO at atmospheric CH$_4$

282 concentrations. Furthermore, both *M. gorgona* MG08 and *M. rosea* SV97 upregulated a high

283 affinity [NiFe] hydrogenase (Hhy), and *M. gorgona* MG08 a molybdenum-dependent carbon

284 monoxide dehydrogenase (CODH), when exposed to air. These differences in enzyme

285 expression patterns demonstrate different metabolic strategies to grow on air: *M. rosea* SV97

286 and *M. gorgona* MG08 compensate for energy limitation by upregulating enzymes for energy

287 conservation from H$_2$ or H$_2$ and CO, while *M. palsarum* NE2 compensates for the limitation by

288 upregulating pMMO. The Hhy increase is similar to the Hhy increase in *Mycobacterium*

289 *smegmatis* enabling long-term persistence after carbon limitation[46]. The observed

290 upregulation of pMMO, Hhy, or CODH implies that the strains allocate resources to increase

291 their a_A^0 (specific apparent affinity) for the respective trace gases as adaptations to growth on

292 air. Additionally, the distinct strategies to grow on air by the closely related strains *M. gorgona*

293 MG08 and *M. palsarum* NE2 suggest niche differentiation between atmMOB in nature.

294 Furthermore, our observations of the consumption of multiple trace gases by atmMOB and

295   adjusted resource allocation for trace gas uptake driven by changes in CH_4 concentration, are

296   in line with earlier observations of a negative correlation between soil H_2 concentrations and

297   the uptake of atmospheric CH_4[47]. Despite the ability of *M. rosea* SV97 and *M. palsarum* NE2

298   to oxidize CO (Fig. 1A), we were not able to determine the responsible protein complex(es) or

299   the corresponding gene expression responses. In *M. rosea* SV97, candidate genes for CODH

300   were identified by blasting the genome sequences against a non-redundant CODH sequence

301   database created by using the Identical Protein Groups resource[48] (Supplementary table S11).

302   However, not all the potential CODH subunits necessary to form a functional CODH were

303   detected in the proteomes. The same blast-based approach did not result in potential

304   candidate genes that could encode a functioning CODH in *M. palsarum* NE2 (Supplementary

305   table S10). Thus, our results indicate that distantly related or previously undiscovered

306   enzymes catalyze atmospheric CO oxidation in *M. palsarum* NE2.

307   The expression of the methanol dehydrogenase (MDH), which catalyzes the second step in

308   CH_4 oxidation, the oxidation of methanol (CH_3OH) to formaldehyde (CH_2O), matched the

309   pMMO expression patterns for all three strains. This indicates a close interaction between

310   these two enzymes as previously suggested for *Methylococcus capsulatus*[49]. MDHs were

311   upregulated at high CH_4 concentrations by *M. gorgona* MG08 and *M. rosea* SV97, but not by

312   *M*. *palsarum* NE2 (Fig. 4). Only the putative lanthanide-dependent methanol dehydrogenase

313   (XoxF) of *M*. *palsarum* NE2 did not follow the pMMO pattern.

314   Formaldehyde is a key intermediate of both catabolism and anabolism in methanotrophs. The

315   enzymes involved in the catabolic oxidation of formaldehyde via methylene-

316   tetrahydromethanopterin (-H_4MPT), methenyl-H_4MPT, and formyl-H_4MPT to formate ($CHOO^-$

317   ), were downregulated in the air treatment of the three strains (Fig. 4). The toxic and highly

318   reactive formaldehyde condenses spontaneously to methylene-H_4MPT[50] and

319   methylenetetrahydrofolate (H_4F)[51] and thus, higher concentrations of formaldehyde

320   activating enzymes (Fae) may not be required for the oxidation of formaldehyde during

321   growth on air. This may explain why enzymes involved in formaldehyde oxidation, despite the

322   upregulation of pMMO and MDH, were downregulated in *M. palsarum* NE2 at atmospheric

323   CH_4 concentrations. At high CH_4 concentrations, the upregulation of Fae and downstream

324   enzymes for further oxidation to formate could represent a cellular detoxification mechanism

325   to avoid high cellular formaldehyde concentrations and to increase NADH synthesis[52].

326   Two different formate dehydrogenases (Fdh A and Fdh AB) that catalyze the energy-

327   conserving oxidation of formate to CO_2 were expressed by *M. gorgona* MG08 and M. *palsarum*

328   NE2 (Fig. 4). The metal-free and potentially irreversible NAD^+-dependent Fdh A[33,53] was

329   upregulated at atmospheric CH_4 concentrations in *M. gorgona* MG08 and *M. palsarum* NE2.

330   Possibly, the upregulation of a one-directional enzyme minimizes back-flow of CO_2 to formate

331   at high CO_2 concentrations and thus prevents energy loss by CO_2 reduction. Fdh AB, which was

332   expressed by all three strains, is molybdopterin-dependent and homologous to the reversible

333   Fdh found in *Rhodobacter capsulatus*[54]. It catalyzes, in addition to formate oxidation, the

334   reduction of CO_2 to formate[54,55]. The Fdh AB was upregulated in all three strains at high CH_4

335   concentration. Under these conditions, when excess reducing power is available, the Fdh AB

336   might enable the reduction of CO_2 for carbon assimilation via the reductive glycine pathway

337   and serine cycle, but further investigations are needed to test whether CO_2 reduction to

338   formate truly occurs in atmMOB.

Comparative proteomics: Carbon assimilation

340 Carbon assimilation was downregulated in all three strains at atmospheric CH_4 concentrations.

341 In the three strains, formaldehyde can condense with H_4F to methylene-H_4F and then be

342 further assimilated through the glycine cleavage system and serine pathway. Additionally,

343 formaldehyde can be first oxidized via the H_4MPT-mediated pathway to formate and then

344 enter, instead of being oxidize to CO_2, the H_4F-mediated reductive pathway to methylene-H_4F

345 (Fig. 4). The increased expression of enzymes involved in the reduction of formate via formyl-

346 H_4F and methenyl-H_4F to methylene-H_4F in the high CH_4 treatment indicates an enhanced

347 investment into carbon assimilation. Since formaldehyde spontaneously condenses with H_4F

348 to methylene-H_4F, the upregulated enzymes for H_4F-mediated formaldehyde oxidation could

349 also contribute to formate formation. However, experiments with *Methylobacterium*

350 *extorquens* demonstrated that formaldehyde oxidation occurred through its H_4MPT-mediated

351 pathway while the reductive pathway via formate to methylene-H_4F represented the major

352 assimilatory flux[52,56]. Therefore, we consider this as the most likely explanation for the

353 expression patterns in *M. gorgona* MG08, *M. rosea* SV97, and *M*. *palsarum* NE2.

354 The glycine cleavage/synthase system (GCS)[57,58] catalyzes the oxidative cleavage of glycine to

355 NADH, NH_3, CO_2, and a methylene group. It also catalyzes the reverse reaction, the synthesis

356 of glycine from NADH, NH_3, CO_2, and methylene-H_4F. The upregulation of the GCS by *M. rosea*

357 SV97 and *M*. *palsarum* NE2 at high CH_4 concentrations suggests an increased synthase activity

358 and thus increased carbon assimilation, corresponding to the overall upregulation of the

359 reductive glycine pathway (Fig. 4). However, we lack a plausible explanation for the increased

360 expression of the GCS by *M. gorgona* MG08 at atmospheric CH_4 concentrations.

361 As the next step in carbon assimilation, the bidirectional serine hydroxymethyltransferase

362 (GlyA) condenses glycine with methylene-H_4F to serine, representing the first step of the

363 serine cycle (Fig. 4). The enzymes involved in the serine cycle were upregulated by the strains

364 when exposed to high CH_4 concentrations, indicating investment into carbon assimilation at

365 this condition.

366 Comparative proteomics: Electron transport chain

367 The relative abundances of protein complexes involved in the electron transport chain for ATP

368 synthesis was lower in atmospheric CH_4 compared to the high CH_4 treatment (Supplementary

369 Fig. 5). The NADH-quinone oxidoreductase, cytochrome c oxidase, and the ATP synthase were

370 all highly expressed at high CH_4 concentrations indicating increased investment into energy

371 conservation at high substrate supply. We propose that to overcome energy limitations when

372 exposed to air, all three strains upregulate the expression of enzymes for the oxidation of at

373 least one trace gas to maximize uptake rates and energy yield, while investments in energy

374 conservation and energy-intensive carbon assimilation are reduced. This reduced investment

375 in assimilation is in line with the low concentrations of the trace gases, low uptake rates, and

376 overall slow growth of atmMOB when incubated with air as energy and carbon source[33].

377

Specific affinity

379   The a_A^0 for CH₄, expressed as the fraction of $V_{max(app)}$ and $K_{m(app)}$, directly represents the

380   capacity to oxidize CH₄ at low concentrations. To test if the pMMO upregulation in *M.*

381   *palsarum* NE2 and downregulation in *M. gorgona* MG08 at atmospheric CH₄ concentrations

382   translate into a higher a_A^0 for CH₄ by *M. palsarum* NE2 compared to *M gorgona* MG08, we

383   measured the a_A^0 of the two strains when grown on air. To avoid increases in the apparent half

384   saturation constant ($K_{m(app)}$) estimates at high CH₄ concentrations[59], we pre-incubated the

385   cultures on filters floating on carbon-free medium for five months with air as sole energy and

386   carbon source.

387   By measuring the CH₄ oxidation per cell and hour at different CH₄ concentrations, we

388   estimated a $K_{m(app)}$ of 48.54 nM CH₄ and a $V_{max(app)}$ of 4.91 x 10^{-8} nmol cell^{-1} h^{-1} resulting in a a_A^0

389   of 1.01 x 10^{-9} L cell^{-1} h^{-1} for *M. gorgona* MG08 (Supplementary Fig. 6). A $K_{m(app)}$ of 48.54 nM

390   CH₄ is within the range of the $K_{m(app)}$ values measured for fresh oxic soils reported by Bender

391   and Conrad (30 – 51 nM)[13], which led to the theory that atmMOB are oligotrophs with a high

392   affinity for CH₄. This is the first observation of a methanotroph in pure culture showing a $K_{m(app)}$

393   that is in the range of the low $K_{m(app)}$ values measured in upland soils[25,60]. Our a_A^0 estimate for

394   *M. gorgona* MG08 (1.01 x 10^{-9} L cell^{-1} h^{-1}) is approximately five times higher than the a_A^0

395   reported by Tveit et al. 1.95 ×10^{-10} L cell^{-1} h^{-1} [33]. However, the $K_{m(app)}$ and $V_{max(app)}$ reported by

396   Tveit et al. amount 4905 nM and 95.4 x 10^{-8} nmol cell^{-1} h^{-1}, respectively, values approximately

397   100 and 20 times higher than in the current study. These differences might derive from the

398   use of liquid cultures pre-incubated at 20% CH₄ by Tveit et al. Such a high CH₄ concentration

399   could have influenced the cellular pMMO concentration, as indicated by the pMMO

400   upregulation by *M. gorgona* MG08 at 1000 p.p.m.v. CH₄ described above (Fig. 4), and thus

401   increased the $K_{m(app)}$ and $V_{max(app)}$ estimates. Dunfield and Conrad reported a similar alteration

402   of $K_{m(app)}$ and $V_{max(app)}$ for *Methylocystis* strain LR1 after comparing starved cells to cells

403   exposed to 10% CH_4 while the a_A^0 was more constant[59].

404   The $K_{m(app)}$ of *M. palsarum* NE2 was 402.08 nM CH_4, which is approximately eight times higher

405   than the $K_{m(app)}$ of *M. gorgona* MG08. Despite this higher $K_{m(app)}$, the a_A^0 of *M. palsarum* NE2

406   was 3.30 x 10^{-9} L cell^{-1} h^{-1}, three times higher than estimated for *M. gorgona* MG08. This high

407   a_A^0 derives from its substantially higher $V_{max(app)}$ of 133 x 10^{-8} nmol cell^{-1} h^{-1} (Supplementary

408   Fig. 6) and aligns with the proteomic data. The upregulation of the pMMO at atmospheric CH_4

409   concentrations by *M. palsarum* NE2 seem to translate into higher a a_A^0 for CH_4 compared to

410   *M. gorgona* MG08 that downregulated its pMMO. This is also reflected in the CH_4 oxidation

411   rate of *M. palsarum* NE2 at atmospheric concentrations, which surpassed the rate of *M.*

412   *gorgona* MG08, despite having a lower apparent affinity for CH_4 (Supplementary Fig. 6).

413   An apparent affinity of 402.08 nM CH_4 is not considered as high affinity [28,43]. Thus, as both

414   strains grow on air, a high apparent affinity for CH_4 cannot be considered a prerequisite for

415   this lifestyle. The a_A^0 of *M. gorgona* MG08 and *M. palsarum* NE2 are approximately equally

416   high and three times higher, respectively, than the recently reported a_A^0 of

417   *Methylotuvimicrobium buryatense* 5GB1C[61] and 30 and 100 times higher, respectively, than

418   the a_A^0 of *Methylocystis* sp. SC2 (3.4×10^{-11} L cell^{-1} h^{-1}) [26], the MOB with the fourth highest a_A^0

419   for CH_4 known so far. However, the different experimental setups and CH_4 concentrations

420   used to determine a_A^0 might render the comparisons invalid[26,61]. Nevertheless, our results

421   show that[58] the specific affinity (a_A^0), rather than the affinity ($K_{m(app)}$), is the appropriate model

422   to determine the efficiency of atmospheric CH_4 utilization by atmMOB. This is in line with the

423   work on oligotrophic substrate uptake at low concentrations by Button[62].

424

Growth on nitrogen from air

426   The four atmMOB, *M. gorgona* MG08, *M. palsarum* NE2, *M. rosea* SV97, and *M. aurea* KYG,

427   encode all genes required for dinitrogen (N_2) fixation[33] and grow in nitrogen-free medium at

428   high CH_4 concentrations[34,35,37,63]. To test the potential for growth on nitrogen from air, we

429   incubated filter cultures of these strains with air as the sole energy, carbon, and nitrogen

430   source. The colony formation of all four strains after three months, and trace gas uptake by

431   *M. gorgona* MG08 after one year, demonstrates growth in the absence of bioavailable

432   nitrogen sources in the medium (Fig. 5A, 5B, and Supplementary Fig. 7). This suggests that all

433   four strains can either cover their nitrogen requirements by the fixation of N_2, by the

434   utilization of atmospheric reactive nitrogen during growth on air, or both. While the N_2

435   concentration in air is approximately 78 %, ammonia concentrations, for example, have been

436   observed to range between 0.2 and 24 p.p.b.v.[64]

437   To test for N_2 fixation during growth on air by *M. gorgona* MG08, we incubated filter cultures

438   in air enriched with ^{15}N-N_2 (~23 atom % (at%) of the total N_2) and CH_4, H_2, and CO

439   concentrations fluctuating between 0.03 and 3 p.p.m.v. for two months. Afterwards, we

440   measured cellular $^{15}N_2$ fixation using NanoSIMS (Fig. 5C). All cells (n = 379) measured during

441   NanoSIMS incorporated ^{15}N (Table S15 and S16) indicating that all cells have been active

442   during incubation. This supports the validity of our energy estimations (as mentioned above)

443   and demonstrates growth on trace gases in air. The cellular ^{15}N ranged from 2.99 at% to 61.89

444   at% with an average of 30.79 at% (SD = 7.82 at%) (Supplementary table S15) while the control

445   without $^{15}N_2$ enrichment averaged at 0.37 at% (SD = 0.04) (Supplementary table S16). Since

446   the $^{15}N_2$ in the headspace amounted to approximately 23 at% of the total N_2 during incubation,

447   the ^{15}N average of the cells should not have amounted to more than 23 at%. The high values

448   might issue from bioavailable ^{15}N-species in the $^{15}N_2$ gas used for incubation[65]. Purity tests of

449 the $^{15}N_2$ gas prior to the $^{15}N_2$ fixation experiments revealed only a minor NO_x contamination

450 of 101.59 (nmol ml^{-1}) with a very low ^{15}N fraction of 0.0014 at% and ammonia levels below

451 detection limit (1 nmol ml^{-1}), and thus do not provide indications that contamination can

452 explain our observations. However, even undetectable trace amounts of ^{15}N ammonia

453 contaminating the $^{15}N_2$ might be sufficient to cause high cellular ^{15}N-enrichments given the

454 low amount of biomass of the filter cultures and the long incubation times. Thus, despite not

455 being able to detect any ^{15}N-contaminants, we cannot exclude contamination and thereby

456 cannot confirm N_2 fixation. However, considering the growth on nitrogen-free medium

457 (Supplementary Fig. 7) and the NanoSIMS experiment, we can conclude that *M. gorgona*

458 MG08 either fixes N_2 or trace concentrations of reactive nitrogen species. This demonstrates

459 that atmMOB can cover their nitrogen requirements for growth, in addition to energy and

460 carbon, from air. Additionally, it suggests that atmMOB may not be nitrogen limited under

461 most natural conditions, partially answering the question by Bodelier and Steenberg regarding

462 conditions that can be nitrogen limiting to atmMOB[39].

463

A

Methylocapsa gorgona MG08   *Methylocapsa palsarum* NE2   *Methylocystis rosea* SV97   *Methylocapsa aurea* KYG

B *Methylocapsa gorgona* MG08

C *Methylocapsa gorgona* MG08
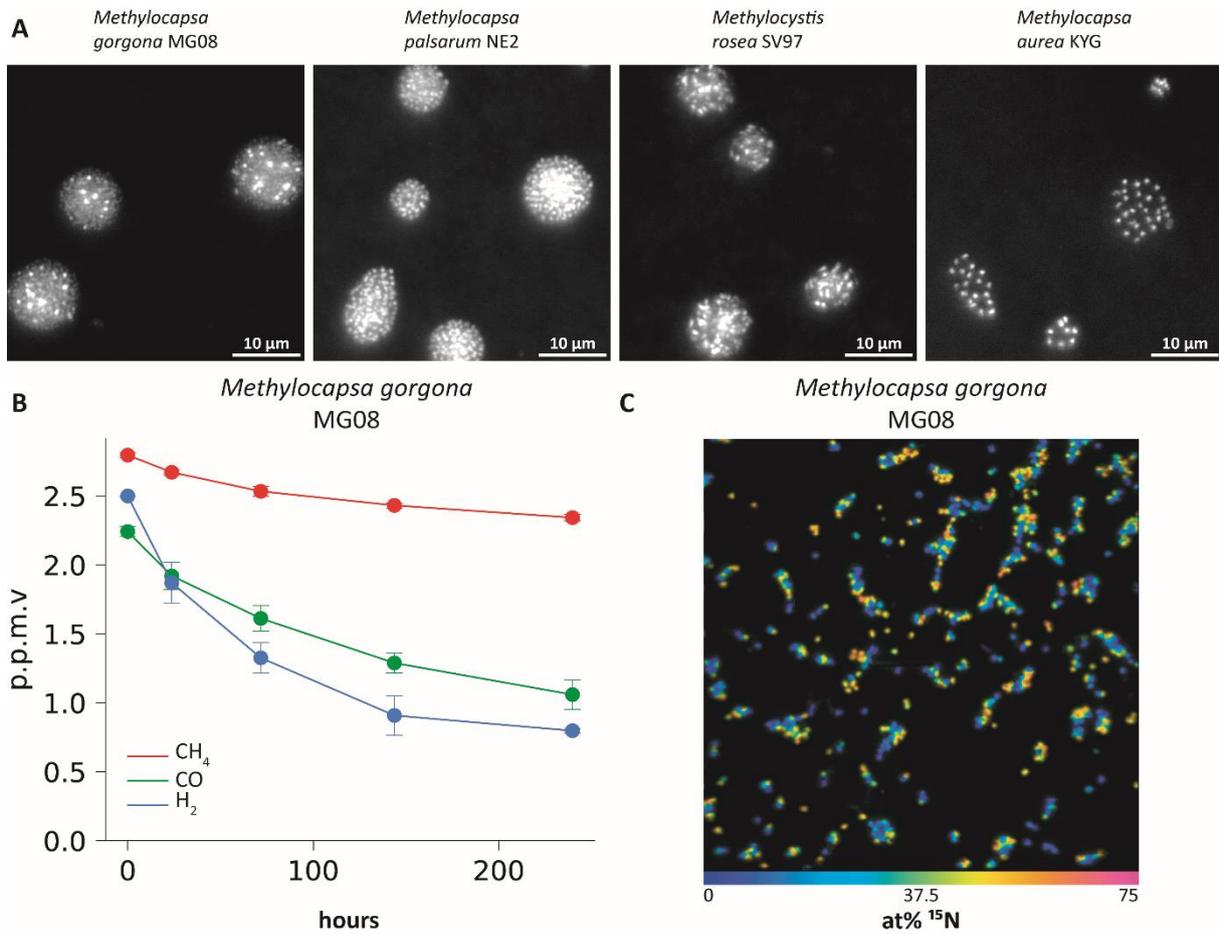
Figure 5. A) Images of SYBR green I stained colonies formed by *M. gorgona* MG08*, M. palsarum* NE2*, M. rosea* SV97, and *M. aurea* KYG after three months of incubation with air as sole energy, carbon, and nitrogen source B) Trace gas oxidation of *Methylocapsa gorgona* MG08 filter cultures after 12 months of incubation with air as sole carbon, energy, and nitrogen source. Error bars indicate standard deviation (n = 4) (Supplementary table S1). C) NanoSIMS visualization of ^{15}N incorporation by *M. gorgona* MG08 after two months of incubation under a ^{15}N$_2$-enriched atmosphere. Fraction values of ^{15}N/(^{14}N + ^{15}N) are given in at%.

Conclusion

Growth on air and enduring oxidation of atmospheric CH_4 is not restricted to members of the clades USCα and γ, but more widespread than previously assumed. Our data shows that former liquid culture-based cultivation approaches lead to an underestimation of the true potential of methanotrophic pure cultures to oxidize atmospheric CH_4. The appearance of atmMOB outside the USCα and γ revises our understanding of the biological atmospheric CH_4 sink and should be considered in future studies. The strain specific oxidation patterns and the estimations of the a_A^0 for CH_4 demonstrate that both the mixotrophic oxidation of atmospheric trace gases and a high a_A^0 for CH_4 are key to obtain sufficient energy for growth on air. The estimated energy requirements for growth of the four atmMOB are substantially lower than the maintenance energy value used as basic premise for an oligotrophic lifestyle of MOB. Additionally, atmMOB seem to cover not only their energy and carbon but also their nitrogen requirements from the atmosphere. This opens a new perspective on physiological limitations of atmospheric trace gas oxidizers and suggests that atmMOB may carry an ideal set of properties needed for pioneering species to initiate primary succession in unfavorable environments. Additionally, the high a_A^0 for CH_4 enables atmMOB to utilize trace concentrations of CH_4 as energy and carbon source for growth while being extremely oligotrophic. This bears the potential for cost-effective and efficient biofiltration of anthropogenic emissions containing CH_4 concentrations far below the lower explosive limit. The common metabolic strategy to grow on air seems to be the downregulation of enzymes involved in energy-intensive processes combined with the upregulation of enzymes for the oxidation of trace gases. However, the differing expression patterns of enzymes for trace gas oxidation and the strain-specific trace gas oxidation patterns indicate that a diverse metabolic repertoire has evolved to enable life on air.

510   Authors contributions

511   T.S., M.M.S. and A.T.T. conceived the study. T.S., A.G.H., J.B., H.S., A.Sch., B.R., E.J.T, O.S., and

512   A.T.T. performed experiments. T.S., H.S., A.Sch., A.S., A.R. and A.T.T. analyzed data. T.S. and

513   A.T.T. created the figures. T.S., H.S., A.Sch., B.R., A.R., A.S., M.P, M.M.S, and A.T.T. contributed

514   to methods. T.S. and A.T.T. wrote the manuscript with inputs from all authors.

515   Competing interests

516   The authors declare no conflict of interest.

517

Methods

Filter-based cultivation with air as the sole carbon and energy source

The strains were pre-incubated in liquid cultures in 100 mL serum bottles containing 10 mL of 10x diluted, EDTA-free NMS medium (DSMZ medium 921 with 10x the iron concentration and 1 µM lanthanum) and a headspace of 20% CH_4 in air. The Fe-EDTA stated in the original DSMZ recipe was substituted with $FeSO_4$. Depending on the strain, the medium was adjusted to a pH of 6.8 (*Methylocapsa gorgona* MG08, *Methylosinus trichosporium* OB3b, *Methylocystis rosea* SV97, *Methylobacter tundripaludum* SV96) or 5.8 (*Methylocapsa aurea* KYG, *Methylocapsa acidiphila* B2, and *Methylocapsa palsarum* NE2). The serum bottles were sealed using butyl rubber stoppers (Chromacol 20-B3P, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and crimp caps, and incubated at 20°C in dark, until the strains were in an exponential growth phase. Strain purity was controlled routinely via microscopy and by confirming the absence of heterotrophic growth on agar plates with a rich medium containing tryptone, yeast extract, and glucose[32]. After reaching exponential growth phase, the strains were diluted with the described medium and filtered on 25 mm polycarbonate (PC) filters (Whatman 10417006, Cytiva, Massachusetts, Marlborough, USA) using a filtration manifold (EQU-FM-10X20-SET, DHI, Hørsholm, Denmark)[28]. The final cell density on the filters amounted approximately 20 cells per photo area (630x magnification). The filters were

541 transferred into Petri dishes containing 10x diluted, EDTA-free NMS medium (DSMZ medium

542 921 with 10x the iron concentration and 1 μM lanthanum) and left floating on the medium

543 with the cells facing upwards. The Petri dishes enable constant ventilation of the filter cultures

544 with ambient air while keeping the cultures sterile. The cultures were incubated at 20°C in

545 dark, with air as the sole energy and carbon source, for at least three months until initiation

546 of experiments. All cultivation steps were carried out under sterile conditions. Before

547 experiments, colony formation and microcolony morphology of the strains was routinely

548 checked via microscopy as previously described[33]. To verify that atmMOB grow on the three

549 trace gases CH_4, CO, and H_2 in air, we incubated *M. gorgona* MG08 cells on filters floating on

550 10x diluted EDTA-free NMS medium in glass bottles sealed with Safety-Caps (JR-S-11011, VICI

551 AG International, Schenkon, Switzerland) under two different atmospheres: One of synthetic

552 air without the trace gases CH_4, CO, and H_2 (Gas composition: 400 p.p.m.v. CO_2 and 20.9% O_2

553 in N_2) and another of ambient air. After an incubation period of 15, 20, and 50 days at 20°C,

554 the growth of cells in the different treatments was compared via microcopy[33] (Supplementary

555 Fig. 2). To create the synthetic air atmosphere, the bottles were gasses as written in "Trace

556 gas oxidation experiments".

557 Trace gas oxidation experiments

558 For all trace gas oxidation experiments, filter cultures of the respective strains, pre-incubated

559 with air as the sole carbon and energy source, were transferred into 250 mL glass bottles

560 containing 50 mL 10x diluted EDTA-free NMS medium (DSMZ medium 921 with 10x the iron

561 concentration and 1 μM lanthanum). The bottles were capped with Safety-Caps (JR-S-11011,

562 VICI AG International). To create a defined atmosphere within the bottles, the headspace of

563 each bottle was flushed for 10 minutes with high-purity synthetic air containing 400 p.p.m.v.

564 CO_2 (HiQ, AGA, Sweden) using a gassing manifold. The headspace pressure was adjusted to

565 approximately 1.05 bar. Then, 1 mL 1000 p.p.m.v. CH_4 in N_2 (HiQ, AGA, Sweden), 1 mL 1000

566 p.p.m.v. H_2 in N_2 (HiQ, AGA, Sweden), and 1 mL 1000 p.p.m.v. CO in N_2 (HiQ, AGA, Sweden)

567 were added to the headspace using a gas tight syringe. To assess the oxidation rate of the

568 respective strains, the cultures were incubated at 20°C and the change in CH_4, H_2, and CO

569 concentrations within bottle headspaces were measured. For each measurement, 2 mL of the

570 gas in the headspace were sampled using a gas tight syringe. The gas samples were analyzed

571 with a gas chromatograph (ThermoScientific Trace 1300, Thermo Fisher Scientific) equipped

572 with a sample loop, a Hayesep column (SU12875, RESTEK, Bellefonte, Pennsylvania, USA), a

573 Molsieve 5A column (PKC17080, RESTEK), a pulsed discharge detector, and a flame ionization

574 detector. A high-quality gas containing 2.5 p.p.m.v. CH_4, 2.5 p.p.m.v. H_2, and 2.5 p.p.m.v. CO

575 in N_2 served as standard (HiQ, AGA, Sweden). To determine the gas concentrations in the

576 bottle headspaces, standard curves were created every day of measurement. The headspace

577 pressure was measured at the end of the experiment using a manometer (LEO1, Keller,

578 Winterthur, Switzerland). The mass of each trace gas was calculated by applying the ideal gas

579 law and adjusted for changes in pressure caused by gas removal during measurement.

580 Cell quantification

581 For quantification, cells from filters were processed as follows: The filters were placed on 150

582 µL 25x SYBR green I (10463252, Thermo Fisher Scientific) with cells facing upwards and

583 incubated for 10 minutes in dark. Next, the cells were washed twice by letting the filters float

584 on milliQ water for 5 minutes in dark. To remove the cells from the filters, the filters were

585 transferred into 5 mL tubes (0030122321, Eppendorf, Hamburg, Germany) containing 2 mL

586 10x diluted solution 1 (DSMZ medium 921), so that the filters sticked to the tube wall and the

587 cells faced inwards[67]. The tubes were vortexed for 10 minutes at medium speed. After

588 vortexing, remaining cells on the filters were washed off and collected by rinsing the filters

589   with 1 mL solution 1 (DSMZ medium 921). The washed filters were dried in dark, and the cell

590   removal controlled via fluorescence microscopy. The 3 mL cell suspension resulting from the

591   cell removal was spiked with 50 µL absolute counting beads (Invitrogen^{tm} CountBright^{tm} Plus,

592   Thermo Fisher Scientific). After, the cells in suspension were immediately counted using a flow

593   cytometer (BD FACSAria III, BD Biosciences, Franklin Lakes, New Jersey, USA). The flow

594   cytometer was set up to capture SYBR Green I (excitation = 498 nm, emission = 522 nm) in the

595   green channel and the Invitrogen^{tm} CountBright^{tm} Plus Absolute Counting Beads (excitation =

596   350 – 810 nm, emission = 385 – 860 nm) in the blue channel. Unstained cells of *M. gorgona*

597   MG08, *M. palsarum* NE2, *M. rosea* SV97, and *M. aurea* KYG were used to define the

598   autofluorescence threshold in the different channels. Size beads (NPPS-4K, Spherotech, Lake

599   Forest, Illinois, USA) were used to draw the forward scatter gate covering events between 0.58

600   and 4 µm (Supplementary Fig. 11). SYBR green I stained cells and absolute counting beads

601   were   counted   to   determine

602   the cell number within filter cultures, using the following equation:

603
$$x_{filter} = \frac{b_{abs}}{b_c} \times \ x_c$$

604   Where x_{filter} is the absolute cell number of the filter culture, b_{abs} the number of counting

605   beads added, b_c the number of counting beads counted, and x_c the number of SYBR green I

606   stained cells counted.

607   Cell-specific oxidation rates and energy calculation

608   The gas-specific oxidation rates of the different strains at atmospheric CH$_4$, H$_2$, and CO

609   concentrations and pseudo first order kinetics were calculated and tested as described by

610   Tveit et al[32]. The oxidation rate per cell was calculated by dividing the oxidation rate of the

611   filter culture by the corresponding cell number. The Gibbs free energy changes ($\Delta_r G$) for the

612 following reactions: $CH_4 + 2O_2 \rightarrow 2H_2O + CO_2$, $2H_2 + O_2 \rightarrow 2H_2O$, $2CO + O_2 \rightarrow 2CO_2$, at

613 20°C, 1.013 bar absolute, and atmospheric concentrations of CH_4 (1.87 p.p.m.v.), H_2 (0.5

614 p.p.m.v.), and CO (0.2 p.p.m.v.) amount to -797.4 kJ mol^{-1}, -236.8 kJ mol^{-1}, and -199.9 kJ mol^{-1}

615 respectively. The values are based on the values for Gibbs free energy of formation

616 found in literature[32,68] and the following equation:

622 $$\Delta_r G = \Delta_r G° + RT ln Q_r$$

617 where $\Delta_r G°$ is the Gibbs free energy change at standard conditions, R the gas constant, T the

618 temperature, ln the natural logarithm, and Q_r the reaction quotient. The estimates of energy

619 yield per cell were obtained from the trace gas oxidation per cell and the $\Delta_r G$. The energy

620 yield from trace gas oxidation per carbon mol of biomass and hour (kJ C-mol^{-1} hour^{-1}) was

621 estimated including the strain specific carbon content and dry weight as previously shown[26].

623 Cellular dry mass and carbon content estimations

624 The strain-specific cellular dry mass was determined by measuring single-cell buoyant mass

625 distributions of the strains in H_2O-based and deuterium oxide (D_2O)-based solutions of

626 phosphate saline buffer (PBS) using a suspended microchannel resonator (SMR)[69,70]. To do so,

627 filter cultures of *M. gorgona* MG08, *M.* rosea SV97, *M. palsarum* NE2, and *M.* aurea KYG were

628 incubated on 10x diluted, EDTA-free NMS medium (DSMZ medium 921 with 10x the iron

629 concentration and 1 µM lanthanum) under an atmosphere of 1000 p.p.m.v. CH_4 in air for three

630 weeks. After, cells of filter cultures were fixed by incubating the filters on 150 µl formaldehyde

631 in 1×PBS (4% w/v) for an hour at room temperature. The cells were washed twice by letting

632 the filters float on water for 5 minutes. The fixed cells were harvested as described in "Cell

633 quantification". The resulting cell suspension was stored at 4°C until measurements of the

634 buoyant cell mass. For the measurements, two aliquots with the same volume of each cell

635 suspension were created. The water of the aliquots was replaced by H_2O-based (1x PBS in H_2O)

636   and D₂O-based (1x PBS in 9:1 D$_2$O:H$_2$O) solutions of known density (1.0043 g cm^{-3} and 1.1033

637   g cm^{-3} at 20°C, respectively). To do so the aliquots were dried in a vacuum concentrator at

638   37°C. After, the cells of one of the two aliquots were resuspended in 50 µl of the H$_2$O-based

639   solution. The cells of the second aliquot were resuspended in 50 µl of the D$_2$O-based solution.

640   The buoyant mass of the cells in the aliquots were measured with a SMR (LifeScale, Affinity

641   Biosensors, Santa Barbara, California, USA). The precision and accuracy of the SMR was

642   verified by creating calibration curves using NIST-certified polystyrene beads (ThermoFisher

643   Scientific) as performed previously[71].

644   The buoyant mass data were exported from the LifeScale instrument and further analyzed

645   using the Python 3[72] packages Pandas[73], Matplotlib[74], and Seaborn[72,75] (Supplementary Fig.

646   10). The dry mass of the strains was calculated as described[69] using the median of the single-

647   cell buoyant mass distributions in H$_2$O-based and D$_2$O-based solutions and the following

648   equation:

649
$$m_{dry} = \frac{\rho_{D_2O} \times m_{b.H_2O} - \rho_{H_2O} \times m_{b.d_2O}}{\rho_{D_2O} - \rho_{H_2O}}$$

650   Where m_{dry} is the dry mass, ρ_{D_2O} the density of the D$_2$O-based solution, $m_{b.H_2O}$ the cell's

651   buoyant mass in the H$_2$O-based solution, ρ_{H_2O} the density of the H$_2$O-based solution, and

652    $m_{b.d_2O}$ the cell's buoyant mass in the D$_2$O-based solution.

653   The cellular carbon contents of the atmMOB were analyzed using an elemental analyzer

654   coupled to an isotope ratio mass spectrometer (EA-IRMS; EA1110 coupled via a ConFlo III

655   interface to a DeltaPLUS IRMS, Thermo Fisher Scientific). Biomass for the carbon content

656   analysis was derived from stirred-tank bioreactor (DASbox® Mini Bioreactor System,

657   Eppendorf) cultures. The cultures were grown in 10x diluted, EDTA-free NMS medium (DSMZ

658   medium 921 with 10x the iron concentration and 1 µM lanthanum) at 80 rpm (marine

659   impeller), 20°C, pH 6.8 (CO_2 controlled) and a gassing rate of 0.24 vessel volumes per minute

660   (6000 p.p.m.v. CH_4 in air) using a microsparger (78530205, Eppendorf). After harvest, cultures

661   were washed three times with milliQ water and lyophilized.

Comparative proteomics

663   For the atmospheric CH_4 treatment, filter-cultures of *M. gorgona* MG08, *M.* rosea SV97 and

664   *M. palsarum* NE2 were incubated with air as the only carbon and energy source for seven

665   months. After, the cells on filters were harvested as described in "Cell quantification",

666   lyophilized, and stored at -80°C until further processing. The 1000 p.p.m.v. CH_4 treatment of

667   the respective strains was processed the same way as the atmospheric CH_4 treatment with

668   the difference that filter cultures had been exposed to approximately 1000 p.p.m.v. CH_4 in air

669   for two weeks before harvest. Samples of lyophilized cells were lysed by sonication in 20 µL

670   buffer containing 4 M urea, 2.5 % sodium deoxycholate (SDC) and 100 mM triethylammonium

671   bicarbonate (TEAB). Samples were sonicated for 25 cycles (1 min on, 30 sec off) with maximum

672   amplitude in a cup horn sonicator with a recirculating chiller (Cup horn: model 413C2, Qsonica,

673   Newtwon, Connecticut, USA; Sonicator: Fisherbrandtm FB705, Thermo Fisher Scientific;

674   Recirculating chiller: model 4905 Qsonica). Then, disulfide bridges were reduced with 1,4-

675   dithiothreitol (DTT) at a final concentration of 5 mM and incubation at 54°C for 30 min.

676   Cysteines were alkylated with 15 mM iodoacetamide (IAA) and incubated for 30 min at room

677   temperature in dark. To remove excess IAA, DTT solution corresponding to a final

678   concentration of 5 mM was added. Calcium chloride solution (final concentration of 1 mM)

679   and 1 µg Lysyl Endopeptidase (125-05061, FUJIFILM Wako Chemicals Europe, Neuss,

680   Germany) were added to the samples and incubated for 5 hours under gentle agitation at 37°C

681   for enzymatic digestion. After, samples were diluted with a buffer containing 100 mM

682 triethylammonium bicarbonate (TEAB) and 1 mM $CaCl_2$ to lower the urea and sodium

683 deoxycholate (SDC) concentration to 1 M and 0.65 % v/v, respectively, resulting in a final

684 sample volume of 80 µL. For digestion, 2 µg trypsin (V511A, Promega, Wisconsin, USA) were

685 added and the samples incubated on a gently agitated shaker at 37°C for 16 hours. After

686 digestion, SDC was precipitated by adding 50% formic acid to the sample (final concentration

687 of 2.5% v/v). Samples were then incubated for 10 min and centrifuged at 16200 rcf for 15 min.

688 Supernatants containing peptides were transferred to low-protein-binding tubes. The

689 peptides were concentrated and cleaned up using DPX C18 pipette tips (DPX Technologies,

690 XTR tips 10 mg C18AQ 300Å) on a Tecan Fluent pipetting robot (Tecan Group Ltd., Männedorf,

691 Switzerland). Purified peptide samples were dried in a vacuum concentrator and dissolved in

692 12 µL 0.1% formic acid. Peptide concentrations were measured on a spectrophotometer

693 (Nanodrop ONE, Thermo Fisher Scientific) at 205 nm. 0.25 µg peptides per sample were loaded

694 onto a liquid chromatograph (EASY-nLC1200, Thermo Fisher Scientific) equipped with an

695 EASY-Spray column (C18, 2µm, 100 Å, 50µm, 50 cm). Peptides were fractionated using a 5 –

696 80% acetonitrile gradient in 0.1 % formic acid over 120 min at a flow rate of 300 nL min^{-1}. The

697 separated peptides were analyzed using a mass spectrometer (Orbitrap Exploris 480, Thermo

698 Fisher Scientific). Data was collected in data dependent mode using a Top40 method.

699 Annotated genomes of the three strains downloaded from MicroScope[76] served as databases

700 for the CHIMERYS-based data search using Proteome Discoverer 3.0. Normalized abundances

701 (scaling mode: On All Average) of proteins were further processed via Perseus[77]. Normal

702 distribution of Log2 fold transformed data was visually screened using histograms. Proteins

703 were filtered using a threshold of at least three valid values in at least one treatment (n = 4

704 per treatment). Proteins that passed the filtering were included in downstream analyses.

705 Missing values were imputed from normal distribution using default settings (width = 0.3,

706 down shift = 1.8). The Pearson correlation coefficient ranged from $\rho = 0.918$ to $\rho = 0.989$

707 between replicates and from 0.68 to 0.799 between replicates of the different treatments.

708 Imputed protein abundance results of the 1.9 p.p.m.v. and 1000 p.p.m.v. treatments were

709 used for correspondence analysis (CA). CA was conducted in R[78] using the "ca" function of R

710 package "ca"[79]. The top 10% of proteins that contributed most to the inertia of the CA's first

711 dimension were extracted using the function "get_ca_row" of the R package "factoextra"[80].

712 To map the abundances and the hierarchical EggNOG[45] annotations of the top 10% proteins,

713 the EggNOG annotations of the three strains were downloaded from MicroScope. For the in-

714 depth analysis of trace gas oxidation, carbon assimilation, and the electron transport chain,

715 the differences between treatments were tested using a two-sided t-test (s0 = 2) and

716 permutation-based false discovery rate (FDR = 0.01). From the z-score normalized results, the

717 proteins involved in trace gas oxidation, carbon assimilation via the serine cycle, and the

718 electron transport chain were selected for further analysis using Python 3[72]. The core carbon

719 and energy metabolism of *M. rosea* SV97 and *M. palsarum* NE2 were reconstructed manually

720 using MicroScope annotations, the published metabolism entries of *M. gorgona* MG08[33],

721 KEGG[81], and protein BLAST searches[82]. To conduct a thorough screening for putative carbon

722 monoxide dehydrogenase subunits a blast-searchable database was constructed out of all

723 sequences retrieved from the NCBI Identical Protein Groups resource[48] (accessed May, 30th

724 2023) using the search term "carbon monoxide dehydrogenase" (54076 amino acid sequence

725 entries). Entries with an amino acid sequence length <30 were removed from the database

726 prior to the blastp search. The annotated genomes of *M. rosea* SV97 and *M. palsarum* NE2,

727 downloaded from MicroScope, were used as blastp queries (default settings, -outfmt 6

728 including slen). After a pre-filtering removing hits with an E-value >1 and an alignment length

729   <50% of the aligned database entry, the blastp output tables were manually evaluated

730   (Supplementary table S10-11).

731   Specific affinity

732   Filter cultures of *M. gorgona* MG08 and *M. palsarum* NE2 were pre-incubated on 10x diluted,

733   EDTA-free NMS medium (DSMZ medium 921 with 10x the iron concentration and 1 µM

734   lanthanum) for five months with air as sole energy and carbon source. The filter cultures were

735   transferred into 250 mL glass bottles containing 250 mL of the mentioned medium. The glass

736   bottles were capped with Safety-Caps (JR-S-11011, VICI AG International). For each strain, the

737   CH_4 concentrations in the 50 mL headspace were adjusted to approximately 1.9, 14, 30, 70,

738   and 175 p.p.m.v. by adding 0 – 2.5 mL 1000 or 5000 p.p.m.v. CH_4 in N_2 (HiQ, Linde, Sweden).

739   The headspace pressure of all bottles was adjusted to approximately 1.1 bar using air. After,

740   the strains were incubated for 48 hours at 20°C. The change in CH_4 was measured at

741   incubation start, after 24 hours and 48 hours as described above. Standard curves were

742   created using 2.5 and 50 p.p.m.v. CH_4 in N_2 (HiQ, AGA, Sweden). At the end of the oxidation

743   experiment, cells in filter cultures were quantified as described above. The change in mass of

744   CH_4 in the headspace was calculated by applying the ideal gas law and adjusted for changes in

745   pressure caused by gas removal during measurement. The mass of dissolved CH_4 at different

746   CH_4 partial pressures in the headspace was calculated by applying the Henry's law solubility

747   constant for CH_4 at 20°C. The Michaelis-Menten CH_4 oxidation kinetics were modelled using

748   the "nls" function of the "nlstools" R package[83], specifying the "michaelis" model and

749   providing start values for $K_{m(app)}$ and $V_{max(app)}$. The a_A^0 was calculated by dividing V_{max} by K_m.

Growth on nitrogen from air

751 Filter cultures of *M. gorgona* MG08, *M. palsarum* NE2, *M. rosea* SV97, and *M. aurea* KYG were

752 pre-incubated on 10x diluted, EDTA-free, and potassium nitrate (KNO_3)-free NMS medium

753 (DSMZ medium 921 with 10x the iron concentration and 1 µM lanthanum) with air as sole

754 energy, carbon, and nitrogen source. After three months, colony formation was checked via

755 microscopy[33]. After 12 months, the activity of *M. gorgona* MG08 was measured as described

756 in the section "Trace gas oxidation experiments" with the only difference that the KNO_3-free

757 medium was used.

758 For the detection of $^{15}N_2$ fixation via NanoSIMS, filter cultures were pre-incubated on 10x

759 diluted, EDTA-free, and potassium nitrate (KNO_3)-free NMS medium with air as sole energy,

760 carbon source, and nitrogen source. After three months, the filters were transferred into 250

761 ml glass bottles containing 50ml of the KNO_3-free NMS medium. The bottles were capped with

762 Safety-Caps (JR-S-11011, VICI AG International) and the headspace of each bottle was flushed

763 for five minutes with compressed air using a gassing manifold. Afterwards, 50 ml of 98+ at%

764 $^{15}N_2$ gas (NLM-363-1-LB, Cambridge Isotopes Laboratories, Tweksbury, Massachusetts, USA)

765 were added using a gas syringe so that the ^{15}N-N_2 in headspace atmosphere amounted

766 approximately 23 at% of the total N_2. The headspace pressure was adjusted to 1.05 bar and

767 0.5 mL 1000 p.p.m.v. CH_4 in N_2 (HiQ, AGA, Sweden), 1 mL 1000 p.p.m.v. H_2 in N_2 (HiQ, AGA,

768 Sweden), and 1 mL 1000 p.p.m.v. CO in N_2 (HiQ, AGA, Sweden) were added to the headspace

769 using a gas syringe. To account for the natural abundance of ^{15}N-N_2, a control without the

770 addition of $^{15}N_2$ gas was prepared accordingly. The filter cultures were incubated at 20°C for

771 two months. The headspace concentration of CH_4, H_2, and CO during incubation was

772 measured once a week as mentioned in the "Trace gas oxidation experiments" section and

773   replenished if the concentration of CH_4, H_2, and CO dropped below 1.9 p.p.m.v., 0.5 p.p.m.v.,

774   0.2 p.p.m.v., respectively.

775   After two months, the cells of the filter cultures were fixed by incubating the filters on 150 µl

776   formaldehyde in 1×PBS (4% w/v) for an hour at room temperature. The cells were washed

777   twice by letting the filters float on water for 5 minutes. The fixed cells were harvested as

778   described in "Cell quantification", lyophilized, and stored at -80°C until further processing. For

779   NanoSIMS analysis the lyophilized cells were resuspended in 20 µl MilliQ water. 10 µl of the

780   cell suspensions were deposited on antimony-doped silicon wafer platelets (7.1 × 7.1 × 0.75

781   mm, Active Business Company, Germany) and dried in air. The following two samples were

782   prepared accordingly for NanoSIMS analysis: i) The unlabeled *M. gorgona* MG08 cells, serving

783   as control for the natural abundance of $^{15}N_2$; and ii) the *M. gorgona* MG08 cells incubated with

784    $^{15}N_2$ gas and trace concentrations of CH_4, H_2, and CO that were analyzed to see whether the

785   strain is capable of fixing atmospheric nitrogen during growth with air as sole energy and

786   carbon source.

787   The NanoSIMS measurements were performed on a NanoSIMS 50 l (Cameca, Gennevilliers,

788   France) at the Large-Instrument Facility for Advanced Isotope Research at the University of

789   Vienna. Before the data acquisition, analysis areas were preconditioned *in situ* by rastering of

790   a high-intensity, defocused Cs^+ ion beam in the following sequence of high and extreme low

791   ion impact energies (HE/16 keV and EXLIE/50 eV, respectively): HE at 25 pA beam current to a

792   Cs+ fluence of 5.0E14 ions cm^{-2}; EXLIE at 400 pA beam current to a fluence of 5.0E16 ions cm^{-2};

793   and HE at 25 pA to a fluence of 5.0E14 ions cm^{-2}. Data were acquired as multilayer image

794   stacks by repeated scanning of a finely focused Cs^+ primary ion beam (c. 80 nm probe size at

795   approx. 2 pA beam current) over areas between 34×34 and 72×72 µm^2 at 512×512-pixel

796   image resolution and a primary ion beam dwell time of 5 ms pixel^{-1}.

797   NanoSIMS images were generated and analyzed with the OpenMIMS plugin[84] in the image

798   processing package Fiji[85]. All images were auto-tracked for compensation of primary ion beam

799   and/or sample stage drift, and secondary ion signal intensities were corrected for detector

800   dead-time and quasi-simultaneous arrival (QSA) of secondary ions, utilizing sensitivity factors

801   ('beta' values) of 1.06, and 1.05 for C_2^-, and CN^- ions, respectively. Regions of interest (ROIs)

802   were defined in the $^{12}C^-$-ion image where each ROI corresponded to an individual cell. Cells

803   touching the border of the image were omitted from the selection.

804   Acquisition cycles of all three analysis areas were reduced to 27 each to improve comparability

805   among the measurements. Regions of interest were analyzed for their ^{15}N content by

806   calculating the average value across acquisition cycles per analysis area, referred to by the

807    $^{15}N/(^{14}N + ^{15}N)$ isotope fraction designated as at% ^{15}N, which was calculated from the $^{12}CN^-$

808   signal intensities via:

809
$$at\% \ ^{15}N = \frac{^{12}C\,^{15}N^-}{^{12}C\,^{15}N^- + ^{12}C\,^{14}N^-}$$

810   The natural abundance of ^{15}N in cellular biomass was inferred from unlabeled cells yielding

811   0.369 ± 0.043 at% (mean ± 1 SD).

812

813   Plotting

814   Plots were created using the Python 3[72] packages Pandas[73], Matplotlib[74], and Seaborn[75] and

815   R packages ggplot2[86]. Figures were finalized using Adobe Illustrator 2023.

References

817   1.   Intergovernmental Panel on Climate Change. The Earth's Energy Budget, Climate
818   Feedbacks and Climate Sensitivity. In: *Climate Change 2021 – The Physical Science Basis*.
819   Cambridge University Press; 2023:923-1054. doi:10.1017/9781009157896.009

820   2.   Mar KA, Unger C, Walderdorff L, Butler T. Beyond CO2 equivalence: The impacts of
821   methane on climate, ecosystems, and health. *Environ Sci Policy*. 2022;134:127-136.
822   doi:10.1016/j.envsci.2022.03.027

823   3.   Ehhalt DH. The atmospheric cycle of methane. *Tellus*. 1974;26(1-2):58-70.
824   doi:10.1111/j.2153-3490.1974.tb01952.x

825   4.   Saunois M, Stavert AR, Poulter B, et al. The Global Methane Budget 2000–2017. *Earth
826   Syst Sci Data*. 2020;12(3):1561-1623. doi:10.5194/essd-12-1561-2020

827   5.   Rigby M, Montzka SA, Prinn RG, et al. Role of atmospheric oxidation in recent methane
828   growth. *Proceedings of the National Academy of Sciences*. 2017;114(21):5373-5377.
829   doi:10.1073/pnas.1616426114

830   6.   Jackson RB, Saunois M, Bousquet P, et al. Increasing anthropogenic methane emissions
831   arise equally from agricultural and fossil fuel sources. *Environmental Research Letters*.
832   2020;15(7):071002. doi:10.1088/1748-9326/ab9ed2

833   7.   Peng S, Lin X, Thompson RL, et al. Wetland emission and atmospheric sink changes
834   explain methane growth in 2020. *Nature*. 2022;612(7940):477-482.
835   doi:10.1038/s41586-022-05447-w

836   8.   Ocko IB, Hamburg SP. Climate consequences of hydrogen emissions. *Atmos Chem Phys*.
837   2022;22(14):9349-9368. doi:10.5194/acp-22-9349-2022

838   9.   Dincer I. Hydrogen 1.0: A new age. *Int J Hydrogen Energy*. 2023;48(43):16143-16147.
839   doi:10.1016/j.ijhydene.2023.01.124

840   10.   Tate KR. Soil methane oxidation and land-use change – from process to mitigation. *Soil
841   Biol Biochem*. 2015;80:260-272. doi:10.1016/j.soilbio.2014.10.010

842   11.   La H, Hettiaratchi JPA, Achari G, Dunfield PF. Biofiltration of methane. *Bioresour
843   Technol*. 2018;268:759-772. doi:10.1016/j.biortech.2018.07.043

844   12.   Harriss RC, Sebacher DI, Day FP. Methane flux in the Great Dismal Swamp. *Nature*.
845   1982;297(5868):673-674. doi:10.1038/297673a0

846   13.   Bender M, Conrad R. Kinetics of CH4 oxidation in oxic soils exposed to ambient air or
847   high CH4 mixing ratios. *FEMS Microbiol Ecol*. 1992;10(4):261-269. doi:10.1111/j.1574-
848   6941.1992.tb01663.x

849   14.   Knief C, Lipski A, Dunfield PF. Diversity and Activity of Methanotrophic Bacteria in
850   Different Upland Soils. *Appl Environ Microbiol*. 2003;69(11):6703-6714.
851   doi:10.1128/AEM.69.11.6703-6714.2003

852   15.   Holmes AJ, Roslev P, McDonald IR, Iversen N, Henriksen K, Murrell JC. Characterization
853   of Methanotrophic Bacterial Populations in Soils Showing Atmospheric Methane

854   Uptake. *Appl Environ Microbiol*. 1999;65(8):3312-3318. doi:10.1128/AEM.65.8.3312-
855   3318.1999

856 16.   Roslev P, Iversen N. Radioactive Fingerprinting of Microorganisms That Oxidize
857   Atmospheric Methane in Different Soils. *Appl Environ Microbiol*. 1999;65(9):4064-4070.
858   doi:10.1128/AEM.65.9.4064-4070.1999

859 17.   Täumer J, Kolb S, Boeddinghaus RS, et al. Divergent drivers of the microbial methane
860   sink in temperate forest and grassland soils. *Glob Chang Biol*. 2021;27(4):929-940.
861   doi:10.1111/gcb.15430

862 18.   Zhao R, Wang H, Cheng X, Yun Y, Qiu X. Upland soil cluster γ dominates the
863   methanotroph communities in the karst Heshang Cave. *FEMS Microbiol Ecol*.
864   2018;94(12):192. doi:10.1093/FEMSEC/FIY192

865 19.   Cai Y, Zhou X, Shi L, Jia Z. Atmospheric Methane Oxidizers Are Dominated by Upland
866   Soil Cluster Alpha in 20 Forest Soils of China. *Microb Ecol*. 2020;80(4):859-871.
867   doi:10.1007/s00248-020-01570-1

868 20.   Pratscher J, Vollmers J, Wiegand S, Dumont MG, Kaster A. Unravelling the Identity,
869   Metabolic Potential and Global Biogeography of the Atmospheric Methane-Oxidizing
870   Upland Soil Cluster α. *Environ Microbiol*. 2018;20(3):1016-1029. doi:10.1111/1462-
871   2920.14036

872 21.   Täumer J, Marhan S, Groß V, et al. Linking transcriptional dynamics of CH4-cycling
873   grassland soil microbiomes to seasonal gas fluxes. *ISME J*. 2022;16(7):1788-1797.
874   doi:10.1038/s41396-022-01229-4

875 22.   Deng Y, Che R, Wang F, et al. Upland Soil Cluster Gamma dominates methanotrophic
876   communities in upland grassland soils. *Science of The Total Environment*. 2019;670:826-
877   836. doi:10.1016/j.scitotenv.2019.03.299

878 23.   Knief C. Diversity and Habitat Preferences of Cultivated and Uncultivated Aerobic
879   Methanotrophic Bacteria Evaluated Based on pmoA as Molecular Marker. *Front
880   Microbiol*. 2015;6(DEC). doi:10.3389/fmicb.2015.01346

881 24.   Dunfield PF, Liesack W, Henckel T, Knowles R, Conrad R. High-Affinity Methane
882   Oxidation by a Soil Enrichment Culture Containing a Type II Methanotroph. *Appl Environ
883   Microbiol*. 1999;65(3):1009-1014. doi:10.1128/AEM.65.3.1009-1014.1999

884 25.   Dunfield PF. The soil methane sink. In: *Greenhouse Gas Sinks*. CABI; 2007:152-170.
885   doi:10.1079/9781845931896.0152

886 26.   Knief C, Dunfield PF. Response and adaptation of different methanotrophic bacteria to
887   low methane mixing ratios. *Environ Microbiol*. 2005;7(9):1307-1317.
888   doi:10.1111/j.1462-2920.2005.00814.x

889 27.   Cai Y, Zheng Y, Bodelier PLE, Conrad R, Jia Z. Conventional methanotrophs are
890   responsible for atmospheric methane oxidation in paddy soils. *Nat Commun*.
891   2016;7(1):11728. doi:10.1038/ncomms11728

892 28.   Conrad R. Soil microorganisms oxidizing atmospheric trace gases (CH4, CO, H2, NO).
893   *Indian J Microbiol*. 1999;39:193-203.

894 29. Tijhuis L, Van Loosdrecht MCM, Heijnen JJ. A thermodynamically based correlation for maintenance gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnol Bioeng*. 1993;42(4):509-519. doi:10.1002/bit.260420415

897 30. Button DK. Differences between the kinetics of nutrient uptake by micro-organisms, growth and enzyme kinetics. *Trends Biochem Sci*. 1983;8(4):121-124. doi:10.1016/0968-0004(83)90232-3

900 31. Degelmann DM, Borken W, Drake HL, Kolb S. Different Atmospheric Methane-Oxidizing Communities in European Beech and Norway Spruce Soils. *Appl Environ Microbiol*. 2010;76(10):3228-3235. doi:10.1128/AEM.02730-09

903 32. Tveit AT, Schmider T, Hestnes AG, Lindgren M, Didriksen A, Svenning MM. Simultaneous Oxidation of Atmospheric Methane, Carbon Monoxide and Hydrogen for Bacterial Growth. *Microorganisms*. 2021;9(1):153. doi:10.3390/microorganisms9010153

906 33. Tveit AT, Hestnes AG, Robinson SL, et al. Widespread soil bacterium that oxidizes atmospheric methane. *Proceedings of the National Academy of Sciences*. 2019;116(17):8515-8524. doi:10.1073/pnas.1817812116

909 34. Wartiainen I, Hestnes AG, McDonald IR, Svenning MM. Methylocystis rosea sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78° N). *Int J Syst Evol Microbiol*. 2006;56(3):541-547. doi:10.1099/ijs.0.63912-0

912 35. Dunfield PF, Belova SE, Vorob'ev A V., Cornish SL, Dedysh SN. Methylocapsa aurea sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus Methylocapsa. *Int J Syst Evol Microbiol*. 2010;60(11):2659-2664. doi:10.1099/ijs.0.020149-0

916 36. Dedysh SN, Khmelenina VN, Suzina NE, et al. Methylocapsa acidiphila gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from Sphagnum bog. *Int J Syst Evol Microbiol*. 2002;52(1):251-261. doi:10.1099/00207713-52-1-251

920 37. Dedysh SN, Didriksen A, Danilova O V., Belova SE, Liebner S, Svenning MM. Methylocapsa palsarum sp. nov., a methanotroph isolated from a subArctic discontinuous permafrost ecosystem. *Int J Syst Evol Microbiol*. 2015;65(Pt_10):3618-3624. doi:10.1099/ijsem.0.000465

924 38. Whittenbury R, Phillips KC, Wilkinson JF. Enrichment, Isolation and Some Properties of Methane-utilizing Bacteria. *J Gen Microbiol*. 1970;61(2):205-218. doi:10.1099/00221287-61-2-205

927 39. Bodelier PLE, Steenbergh AK. Interactions between methane and the nitrogen cycle in light of climate change. *Curr Opin Environ Sustain*. 2014;9-10:26-36. doi:10.1016/j.cosust.2014.07.004

930 40. Zhang T, Zhou J, Wang X, Zhang Y. Poly-β-hydroxybutyrate Production by Methylosinus trichosporium OB3b at Different Gas-phase Conditions. *Iran J Biotechnol*. 2019;17(1):10-16. doi:10.21859/IJB.1866

933 41. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control
934   region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*.
935   1993;10(3):512-526. doi:10.1093/oxfordjournals.molbev.a040023

936 42. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis
937   Version 11. Battistuzzi FU, ed. *Mol Biol Evol*. 2021;38(7):3022-3027.
938   doi:10.1093/molbev/msab120

939 43. Greening C, Grinter R. Microbial oxidation of atmospheric trace gases. *Nat Rev*
940   *Microbiol*. 2022;20(9):513-528. doi:10.1038/s41579-022-00724-x

941 44. Bonk F, Popp D, Weinrich S, et al. Determination of Microbial Maintenance in
942   Acetogenesis and Methanogenesis by Experimental and Modeling Techniques. *Front*
943   *Microbiol*. 2019;10(FEB). doi:10.3389/fmicb.2019.00166

944 45. Cantalapiedra CP, Hernández-Plaza A, Letunic I, Bork P, Huerta-Cepas J. eggNOG-
945   mapper v2: Functional Annotation, Orthology Assignments, and Domain Prediction at
946   the Metagenomic Scale. Tamura K, ed. *Mol Biol Evol*. 2021;38(12):5825-5829.
947   doi:10.1093/molbev/msab293

948 46. Cordero PRF, Grinter R, Hards K, et al. Two uptake hydrogenases differentially interact
949   with the aerobic respiratory chain during mycobacterial growth and persistence.
950   *Journal of Biological Chemistry*. 2019;294(50):18980-18991.
951   doi:10.1074/jbc.RA119.011076

952 47. Piché-Choquette S, Khdhiri M, Constant P. Dose-response relationships between
953   environmentally-relevant H2 concentrations and the biological sinks of H2, CH4 and CO
954   in soil. *Soil Biol Biochem*. 2018;123:190-199. doi:10.1016/J.SOILBIO.2018.05.008

955 48. Agarwala R, Barrett T, Beck J, et al. Database resources of the National Center for
956   Biotechnology Information. *Nucleic Acids Res*. 2018;46(D1):D8-D13.
957   doi:10.1093/nar/gkx1095

958 49. Culpepper MA, Rosenzweig AC. Structure and protein-protein interactions of methanol
959   dehydrogenase from Methylococcus capsulatus (Bath). *Biochemistry*.
960   2014;53(39):6211-6219. doi:org/10.1021/bi500850j

961 50. Escalante-Semerena JC, Wolfe RS. Tetrahydromethanopterin-dependent
962   methanogenesis from non-physiological C1 donors in Methanobacterium
963   thermoautotrophicum. *J Bacteriol*. 1985;161(2):696-701. doi:10.1128/jb.161.2.696-
964   701.1985

965 51. Kallen RG, Jencks WP. The Mechanism of the Condensation of Formaldehyde with
966   Tetrahydrofolic Acid. *Journal of Biological Chemistry*. 1966;241(24):5851-5863.
967   doi:10.1016/S0021-9258(18)96350-7

968 52. Marx CJ, Chistoserdova L, Lidstrom ME. Formaldehyde-detoxifying role of the
969   tetrahydromethanopterin-linked pathway in Methylobacterium extorquens AM1. *J*
970   *Bacteriol*. 2003;185(24):7160-7168. doi:10.1128/JB.185.23.7160-7168.2003

971 53. Moon M, Park GW, Lee J pyo, Lee JS, Min K. Recent progress in formate dehydrogenase
972   (FDH) as a non-photosynthetic CO2 utilizing enzyme: A short review. *Journal of CO2*
973   *Utilization*. 2020;42:101353. doi:10.1016/j.jcou.2020.101353

974 54. Hartmann T, Leimkühler S. The oxygen-tolerant and NAD+-dependent formate
975   dehydrogenase from Rhodobacter capsulatus is able to catalyze the reduction of CO2
976   to formate. *FEBS J*. 2013;280(23):6083-6096. doi:10.1111/febs.12528

977 55. Yu X, Niks D, Mulchandani A, Hille R. Efficient reduction of CO2 by the molybdenum-
978   containing formate dehydrogenase from Cupriavidus necator (Ralstonia eutropha).
979   *Journal of Biological Chemistry*. 2017;292(41):16872-16879.
980   doi:10.1074/jbc.M117.785576

981 56. Crowther GJ, Kosály G, Lidstrom ME. Formate as the Main Branch Point for
982   Methylotrophic Metabolism in *Methylobacterium extorquens* AM1. *J Bacteriol*.
983   2008;190(14):5057-5062. doi:10.1128/JB.00228-08

984 57. Kikuchi G, Motokawa Y, Yoshida T, Hiraga K. Glycine cleavage system: reaction
985   mechanism, physiological significance, and hyperglycinemia. *Proceedings of the Japan
986   Academy, Series B*. 2008;84(7):246-263. doi:10.2183/pjab.84.246

987 58. Claassens NJ, Satanowski A, Bysani VR, et al. Engineering the Reductive Glycine
988   Pathway: A Promising Synthetic Metabolism Approach for C1-Assimilation. In: *Advances
989   in Biochemical Engineering/Biotechnology*. Vol 180. Adv Biochem Eng Biotechnol;
990   2022:299-350. doi:10.1007/10_2021_181

991 59. Dunfield PF, Conrad R. Starvation Alters the Apparent Half-Saturation Constant for
992   Methane in the Type II Methanotroph Methylocystis Strain LR1. *Appl Environ Microbiol*.
993   2000;66(9):4136-4138. doi:10.1128/AEM.66.9.4136-4138.2000

994 60. Knief C, Kolb S, Bodelier PLE, Lipski A, Dunfield PF. The active methanotrophic
995   community in hydromorphic soils changes in response to changing methane
996   concentration. *Environ Microbiol*. 2006;8(2):321-333. doi:10.1111/j.1462-
997   2920.2005.00898.x

998 61. He L, Groom JD, Wilson EH, et al. A methanotrophic bacterium to enable methane
999   removal for climate mitigation. *Proceedings of the National Academy of Sciences*.
1000   2023;120(35). doi:10.1073/pnas.2310046120

1001 62. Button DK. Nutrient-limited microbial growth kinetics: overview and recent advances.
1002   *Antonie Van Leeuwenhoek*. 1993;63(3-4):225-235. doi:10.1007/BF00871220

1003 63. Murrell JC, Dalton H. Nitrogen Fixation in Obligate Methanotrophs. *Microbiology (N Y)*.
1004   1983;129(11):3481-3486. doi:10.1099/00221287-129-11-3481

1005 64. Nair AA, Yu F. Quantification of Atmospheric Ammonia Concentrations: A Review of Its
1006   Measurement and Modeling. *Atmosphere (Basel)*. 2020;11(10):1092.
1007   doi:10.3390/atmos11101092

1008 65. Dabundo R, Lehmann MF, Treibergs L, et al. The Contamination of Commercial 15N2
1009   Gas Stocks with 15N–Labeled Nitrate and Ammonium and Consequences for Nitrogen
1010   Fixation Measurements. Love JB, ed. *PLoS One*. 2014;9(10):e110335.
1011   doi:10.1371/journal.pone.0110335

1012 66. Perez-Riverol Y, Bai J, Bandla C, et al. The PRIDE database resources in 2022: A hub for
1013   mass spectrometry-based proteomics evidences. *Nucleic Acids Res*. 2022;50(D1).
1014   doi:10.1093/nar/gkab1038

1015 67. Couradeau E, Sasse J, Goudeau D, et al. Probing the active fraction of soil microbiomes
1016   using BONCAT-FACS. *Nat Commun*. 2019;10(1):2770. doi:10.1038/s41467-019-10542-
1017   0

1018 68. Dean JA. LANGE'S HANDBOOK OF CHEMISTRY. *Materials and Manufacturing Processes*.
1019   1990;5(4):687-688. doi:10.1080/10426919008953291

1020 69. Feijó Delgado F, Cermak N, Hecht VC, et al. Intracellular Water Exchange for Measuring
1021   the Dry Mass, Water Mass and Changes in Chemical Composition of Living Cells.
1022   Polymenis M, ed. *PLoS One*. 2013;8(7):e67590. doi:10.1371/journal.pone.0067590

1023 70. Cermak N, Becker JW, Knudsen SM, Chisholm SW, Manalis SR, Polz MF. Direct single-
1024   cell biomass estimates for marine bacteria via Archimedes' principle. *ISME J*.
1025   2017;11(3):825-828. doi:10.1038/ismej.2016.161

1026 71. Roller BRK, Hellerschmied C, Wu Y, et al. Single-cell mass distributions reveal simple
1027   rules for achieving steady-state growth. Ruby EG, ed. *mBio*. Published online September
1028   6, 2023. doi:10.1128/mbio.01585-23

1029 72. Van Rossum G, Drake FL. *Python 3 Reference Manual*. CreateSpace; 2009.

1030 73. The Pandas Development T, eam. pandas-dev/pandas: Pandas. Published online
1031   February 2020. doi:org/10.5281/zenodo.3509134

1032 74. Hunter JD. Matplotlib: A 2D Graphics Environment. *Comput Sci Eng*. 2007;9(3):90-95.
1033   doi:10.1109/MCSE.2007.55

1034 75. Waskom M. Seaborn: Statistical Data Visualization. *J Open Source Softw*.
1035   2021;6(60):3021. doi:10.21105/joss.03021

1036 76. Vallenet D, Calteau A, Dubois M, et al. MicroScope: An integrated platform for the
1037   annotation and exploration of microbial gene functions through genomic, pangenomic
1038   and metabolic comparative analysis. *Nucleic Acids Res*. 2019;48(D1):D579-D589.
1039   doi:10.1093/nar/gkz926

1040 77. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for
1041   comprehensive analysis of (prote)omics data. *Nat Methods*. 2016;13(9):731-740.
1042   doi:10.1038/nmeth.3901

1043 78. R Core Team. R: A Language and Environment for Statistical Computing. Published
1044   online 2022. https://www.R-project.org/

1045 79. c ON, Greenacre M. Correspondence Analysis in R, with Two- and Three-dimensional
1046   Graphics: The ca Package. *J Stat Softw*. 2007;20(3). doi:10.18637/jss.v020.i03

1047 80. Kassambara A, Mundt F. factoextra: Extract and Visualize the Results of Multivariate
1048   Data Analyses. Published online April 1, 2020. Accessed October 13, 2023.
1049   http://www.sthda.com/english/rpkgs/factoextra

1050 81. Kanehisa M. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res*.
1051   2000;28(1):27-30. doi:10.1093/nar/28.1.27

1052 82. Altschul S. Gapped BLAST and PSI-BLAST: a new generation of protein database search
1053   programs. *Nucleic Acids Res*. 1997;25(17):3389-3402. doi:10.1093/nar/25.17.3389

1054   83.   Baty F, Ritz C, Charles S, Brutsche M, Flandrois JP, Delignette-Muller ML. A Toolbox for
1055   Nonlinear Regression in *R* : The Package **nlstools**. *J Stat Softw*. 2015;66(5).
1056   doi:10.18637/jss.v066.i05

1057   84.   Gormanns P, Reckow S, Poczatek JC, Turck CW, Lechene C. Segmentation of Multi-
1058   Isotope Imaging Mass Spectrometry Data for Semi-Automatic Detection of Regions of
1059   Interest. Rogers S, ed. *PLoS One*. 2012;7(2):e30576. doi:10.1371/journal.pone.0030576

1060   85.   Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for
1061   biological-image analysis. *Nat Methods*. 2012;9(7):676-682. doi:10.1038/nmeth.2019

1062   86.   Wickham H. *Ggplot2*. Springer International Publishing; 2016. doi:10.1007/978-3-319-
1063   24277-4

1064

1065
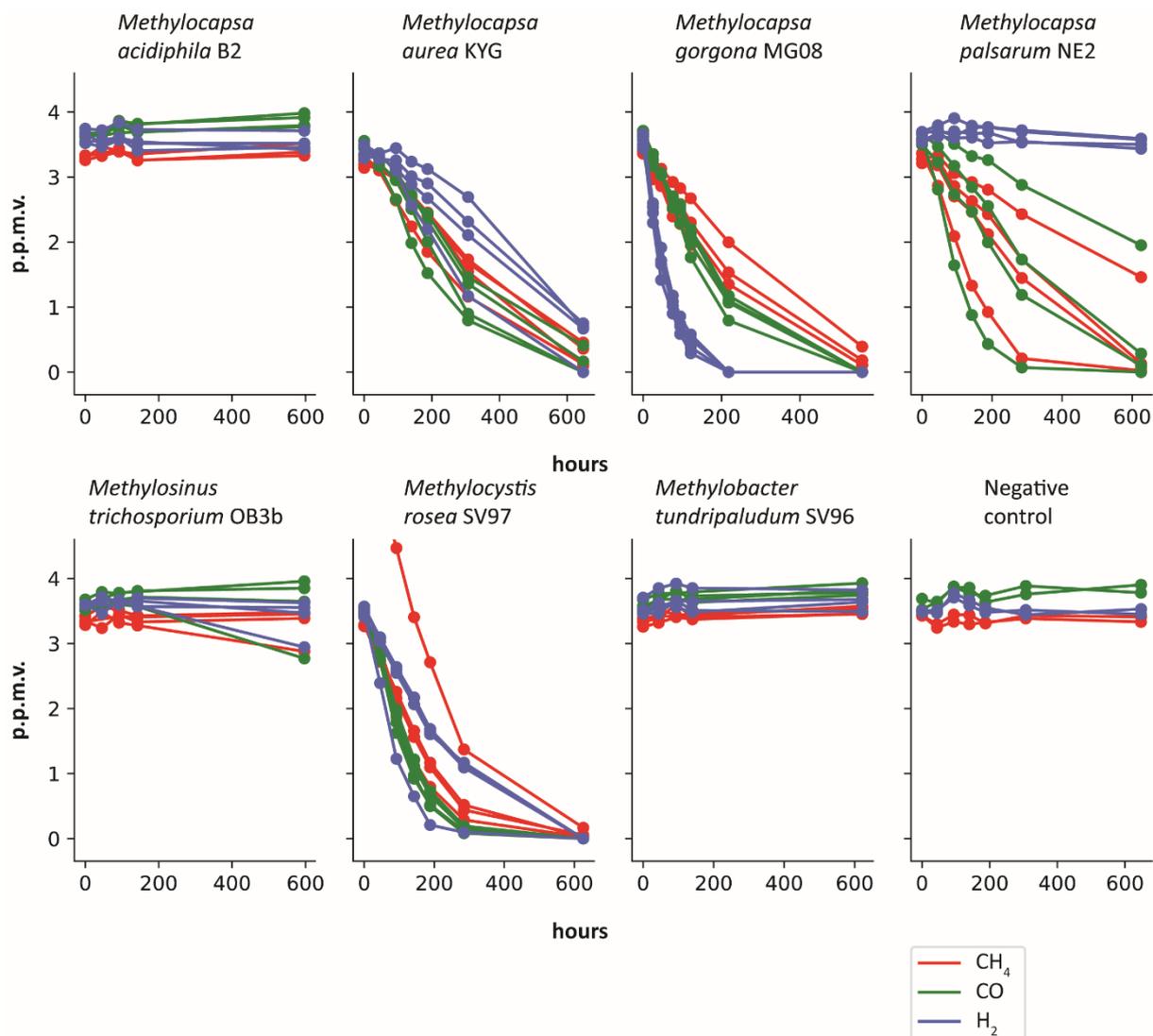
1067

Supplementary Figure 1. CH_4, H_2, and CO oxidation at atmospheric pressure by *M. acidiphila* B2, *M. aurea* KYG, *M. gorgona* MG08, *M. palsarum* NE2, *M. trichosporium* OB3b, *M. rosea* SV97, and *M. tundripaludum* SV96 after 12 months of incubation with air as sole carbon and energy source (Supplementary table S1).
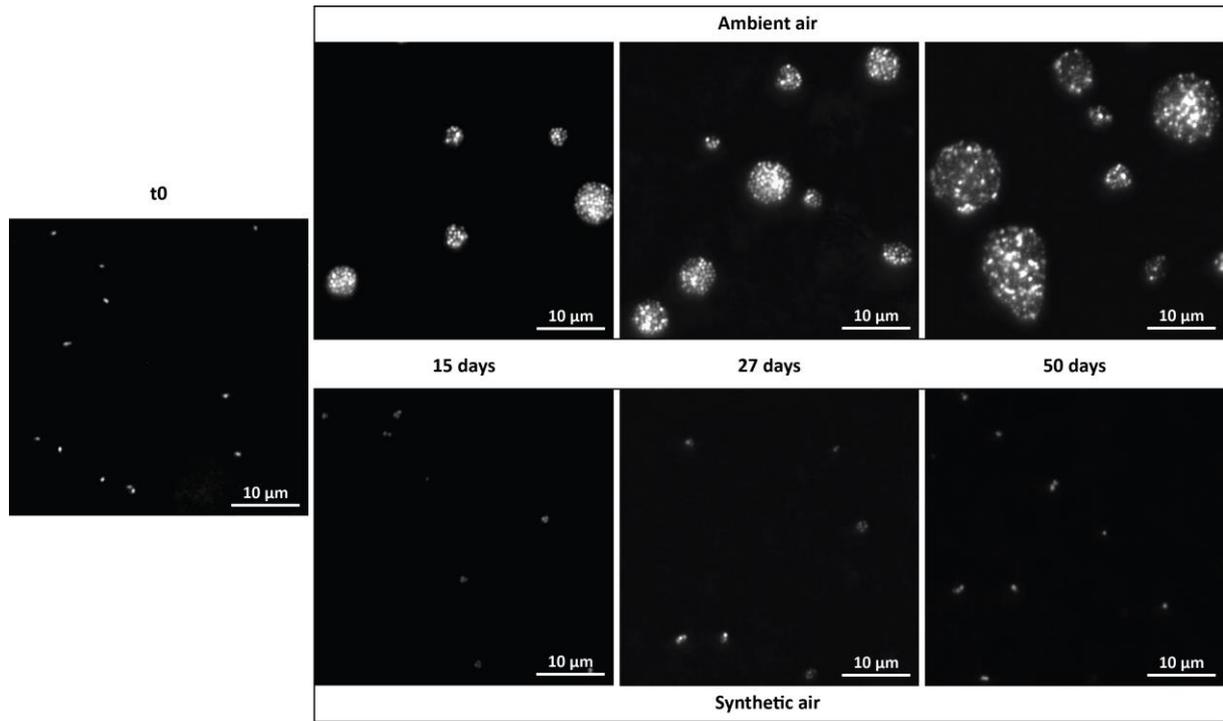
1071

Supplementary Figure 2. SYBR green I stained cells of *M. gorgona* MG08 incubated for 15, 27, and 50 days under an atmosphere of ambient air and an atmosphere of synthetic air without the atmospheric trace gases CH_4, CO, and H_2.
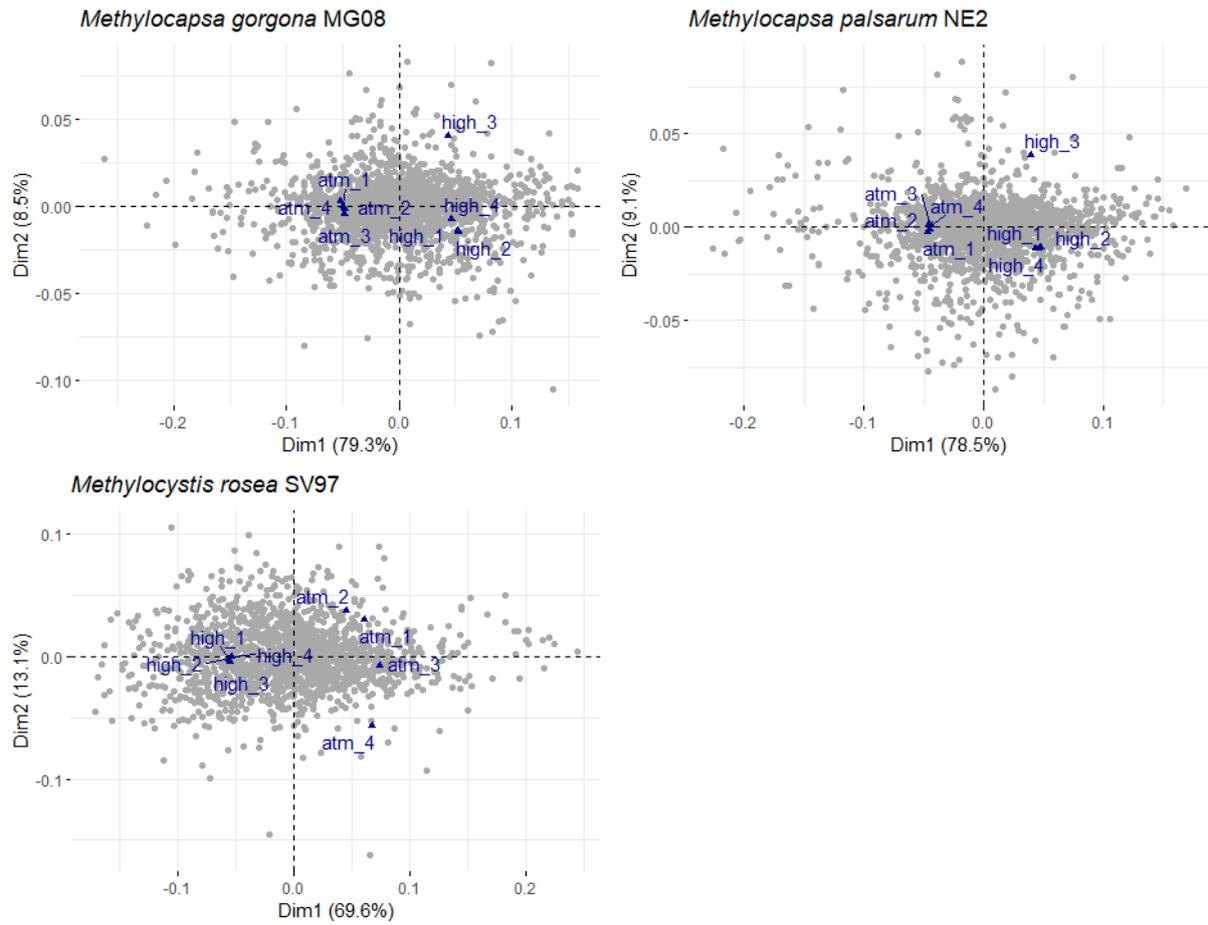
1074

Supplementary Figure 3. Correspondence analysis biplot of protein abundances of *M. gorgona* MG08, *M. palsarum* NE2, and
M. rosea SV97 at 1.9 p.p.m.v. (atm) and 1000 p.p.m.v. (high) methane in air (Supplementary table S6-8). Replicates (n = 4)
incubated at the two CH_4 concentrations are shown in blue. Proteins are shown in grey. Dim – dimension.
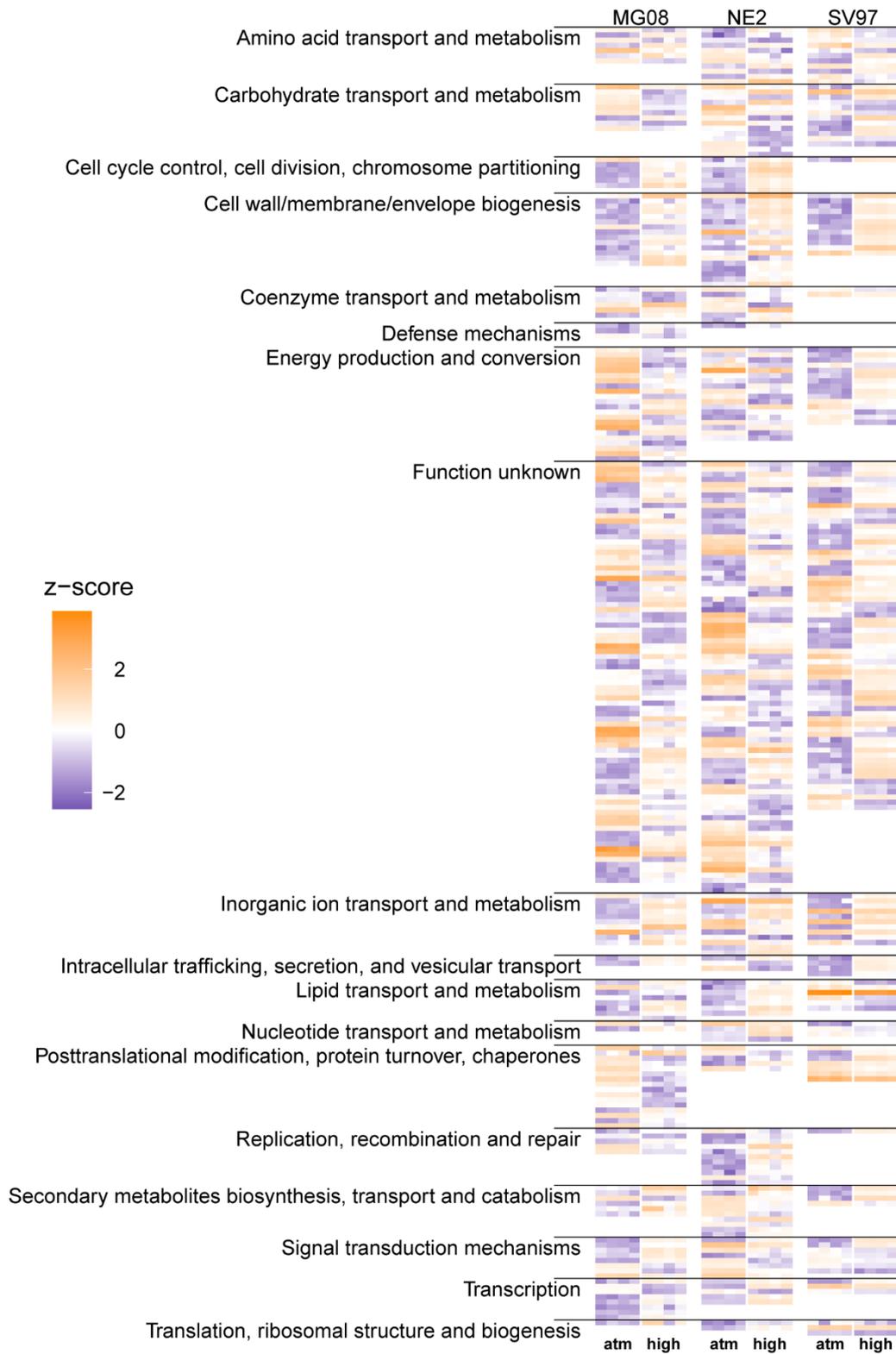
Supplementary Figure 4. Comparative proteomics of *M. gorgona* MG08, *M. rosea* SV97, and *M. palsarum* NE2 exposed to 1000 p.p.m.v. CH$_4$ (high) in air and 1.9 p.p.m.v. CH$_4$ (atm) in air (Supplementary table S12-14). Normalized and standardized expression of top 10% proteins contributing most to the inertia of the correspondence analyses (Supplementary Figure 3). High relative abundance = orange, low relative abundance expression = blue. The proteins are grouped by the hierarchical EggNOG categories.
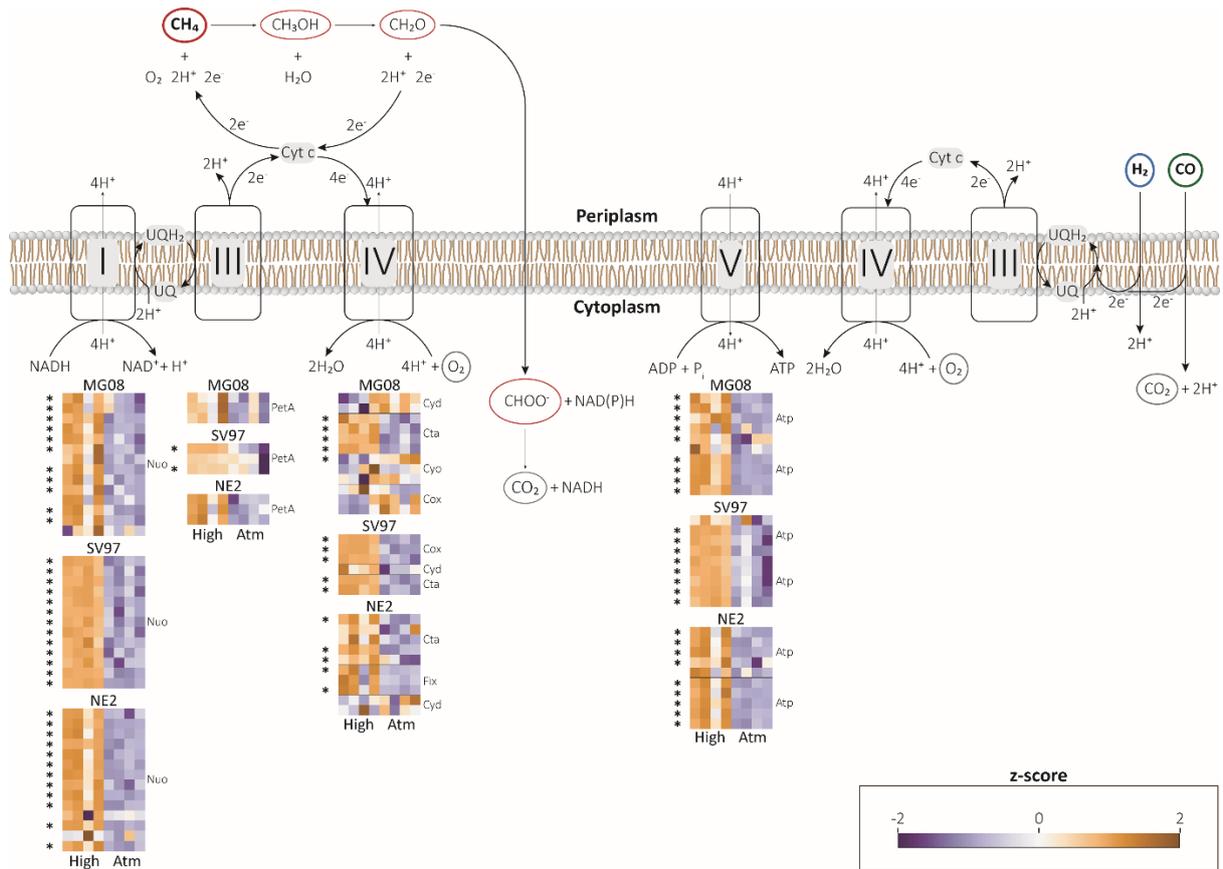
1084

Supplementary Figure 5. Comparative proteomics of *M. gorgona* MG08, *M. rosea* SV97, and *M. palsarum* NE2 exposed to 1000 p.p.m.v. CH$_4$ (High) in air and 1.9 p.p.m.v. (Atm) CH$_4$ in air (Supplementary table S5). Normalized and standardized expression of enzymes involved in the electron transport chain. High relative abundance = orange, low relative abundance expression = blue. * indicates significant difference in expression between treatments (two sided t-test). Horizontal lines in the heatmaps separate operons of enzymes catalyzing the same reaction. I – NADH ubiquinone oxidoreductase, III – ubiquinol-cytochrome c reductase, IV – cytochrome c oxidase, V – ATP synthase.
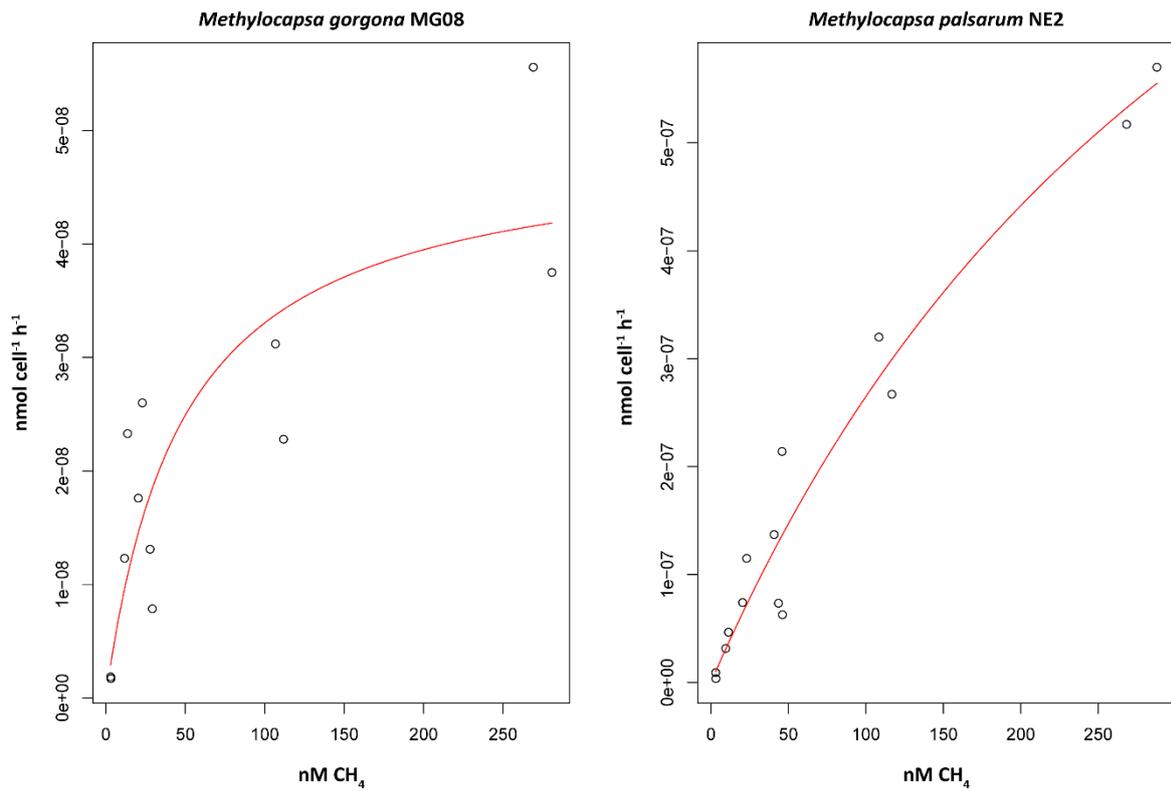
Supplementary Figure 6. Michaelis-Menten hyperbolic curve fitted to the oxidation rate per cell of *M. gorgona* MG08 and *M. palsarum* NE2 at CH_4 concentrations ranging between 3 nM and 287 nM (Supplementary table S9). The nM CH_4 display CH_4 concentrations dissolved in water and correlate with the partial pressure of CH_4 above the water. At 20°C and atmospheric pressure (1.013 bar), 2.98, 50, 100, and 250 nM CH_4 dissolved in water correspond to 1.9, 31.9, 63.7, and 159.3 p.p.m.v. in air.
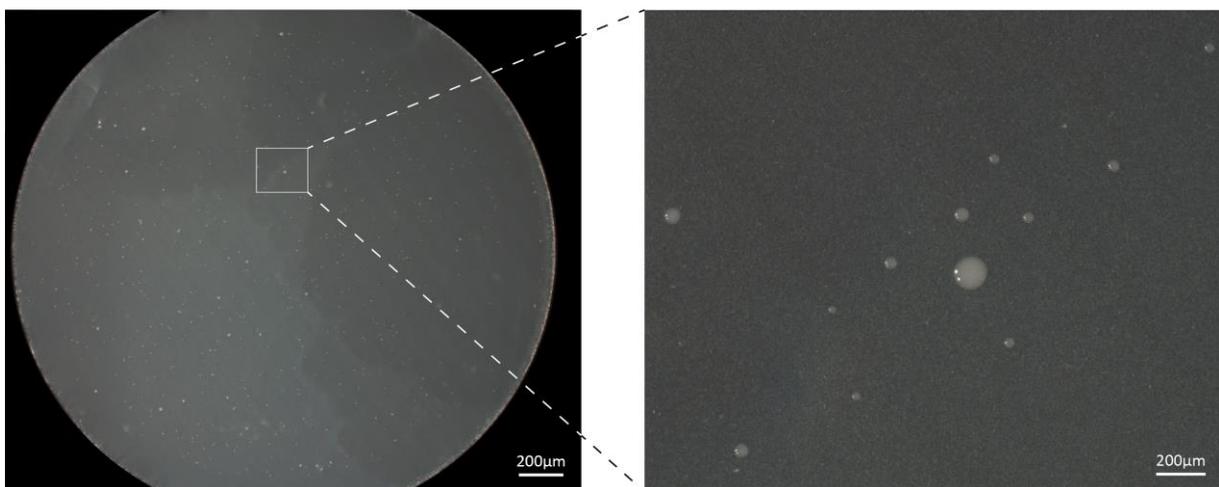
Supplementary Figure 7. Light microscopic image of a *Methylocapsa gorgona* MG08 culture on a polycarbonate filter after 12

months incubation period on a carbon- and nitrogen-free growth medium
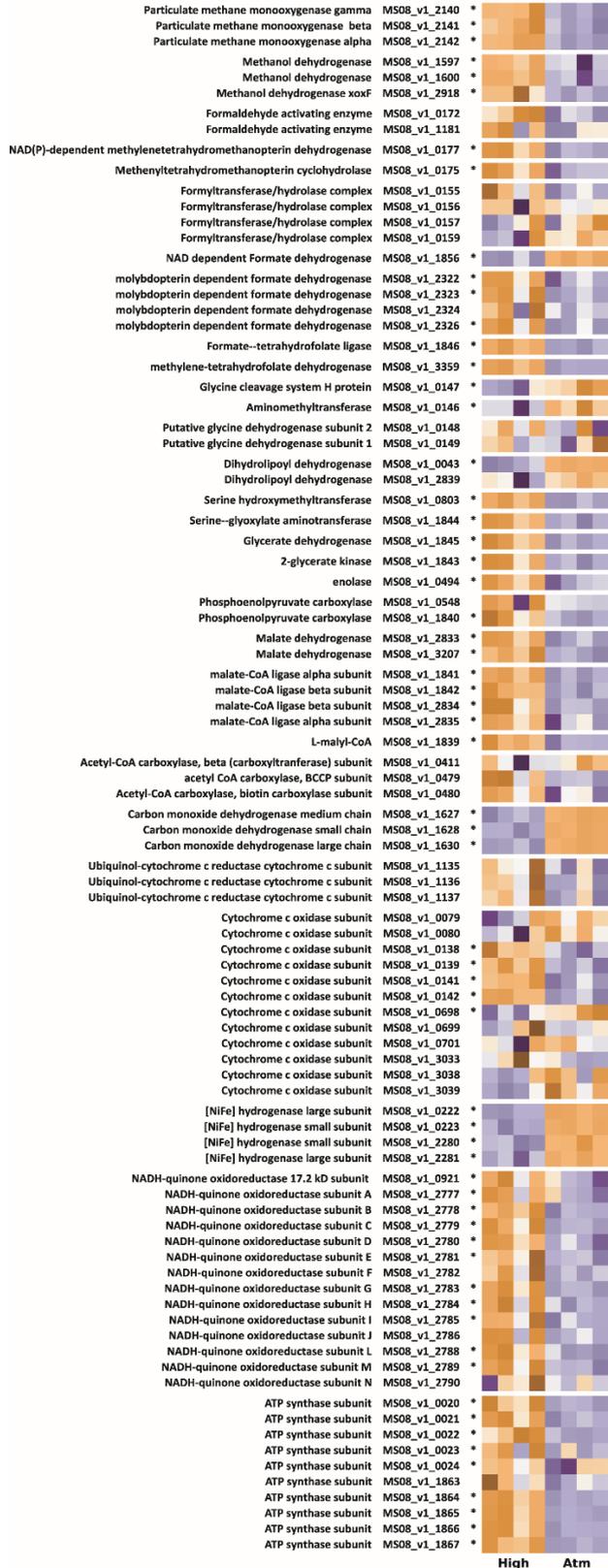
Supplementary Figure 8. Comparative proteomics of *M. gorgona* MG08, *M. rosea* SV97exposed to 1000 p.p.m.v CH$_4$ (High) in air and 1.9 p.p.m.v. (Atm) CH$_4$ in air (Supplementary table S5). Normalized and standardized expression of enzymes involved in the central carbon and energy metabolism is shown. Same heatmaps as in Figure 4 and Supplementary Figure 5 but with protein name and MicroScope accession number. * indicates significant difference in expression between treatments (two sided t-test).

Methylocapsa palsarum NE2

Protein name	Accession
Particulate methane monooxygenase alpha	MPAL_v1_370178 *
Particulate methane monooxygenase beta	MPAL_v1_370179 *
Particulate methane monooxygenase gamma	MPAL_v1_370180 *
Methanol dehydrogenase	MPAL_v1_350017 *
Methanol dehydrogenase	MPAL_v1_350020 *
Methanol dehydrogenase xoxF	MPAL_v1_370060 *
Formaldehyde activating enzyme	MPAL_v1_400110
Formaldehyde activating enzyme	MPAL_v1_460097
NAD(P)-dependent methylenetetrahydromethanopterin dehydrogenase	MPAL_v1_460092
Methenyltetrahydromethanopterin cyclohydrolase	MPAL_v1_460094
Formyltransferase/hydrolase complex	MPAL_v1_460109
Formyltransferase/hydrolase complex	MPAL_v1_460110
Formyltransferase/hydrolase complex	MPAL_v1_460111
Formyltransferase/hydrolase complex	MPAL_v1_460112
NAD dependent Formate dehydrogenase	MPAL_v1_460020 *
molybdopterin dependent formate dehydrogenase	MPAL_v1_460471 *
molybdopterin dependent formate dehydrogenase	MPAL_v1_470008 *
molybdopterin dependent formate dehydrogenase	MPAL_v1_470009 *
molybdopterin dependent formate dehydrogenase	MPAL_v1_470010
Formate--tetrahydrofolate ligase	MPAL_v1_460080 *
methylene-tetrahydrofolate dehydrogenase	MPAL_v1_460413 *
Glycine cleavage system H protein	MPAL_v1_460544 *
Aminomethyltransferase	MPAL_v1_460543
Putative glycine dehydrogenase subunit 2	MPAL_v1_460545 *
Putative glycine dehydrogenase subunit 1	MPAL_v1_460546 *
Dihydrolipoyl dehydrogenase	MPAL_v1_430048
Dihydrolipoyl dehydrogenase	MPAL_v1_470092
Serine hydroxymethyltransferase	MPAL_v1_460398 *
Serine--glyoxylate aminotransferase	MPAL_v1_460082 *
Glycerate dehydrogenase	MPAL_v1_460081 *
2-glycerate kinase	MPAL_v1_460083
enolase	MPAL_v1_470085
Phosphoenolpyruvate carboxylase	MPAL_v1_460087
Phosphoenolpyruvate carboxylase	MPAL_v1_470206 *
Malate dehydrogenase	MPAL_v1_260182
Malate dehydrogenase	MPAL_v1_430053 *
malate-CoA ligase alpha subunit	MPAL_v1_430051
malate-CoA ligase beta subunit	MPAL_v1_430052
malate-CoA ligase beta subunit	MPAL_v1_460085 *
malate-CoA ligase alpha subunit	MPAL_v1_460086 *
L-malyl-CoA	MPAL_v1_460088
acetyl-CoA carboxylase, biotin carboxylase subunit	MPAL_v1_550186
Acetyl CoA carboxylase, BCCP subunit	MPAL_v1_550187 *
Acetyl-CoA carboxylase, beta (carboxyltranferase) subunit	MPAL_v1_560175
Ubiquinol-cytochrome c reductase cytochrome c subunit	MPAL_v1_460461
Ubiquinol-cytochrome c reductase cytochrome c subunit	MPAL_v1_460462
Ubiquinol-cytochrome c reductase cytochrome c subunit	MPAL_v1_460463
Cytochrome c oxidase subunit	MPAL_v1_260166 *
Cytochrome c oxidase subunit	MPAL_v1_260167
Cytochrome c oxidase subunit	MPAL_v1_260169
Cytochrome c oxidase subunit	MPAL_v1_260170 *
Cytochrome c oxidase subunit	MPAL_v1_370160 *
Cytochrome c oxidase subunit	MPAL_v1_370206 *
Cytochrome c oxidase subunit	MPAL_v1_370207
Cytochrome c oxidase subunit	MPAL_v1_370209 *
Cytochrome c oxidase subunit	MPAL_v1_460202
Cytochrome c oxidase subunit	MPAL_v1_460203
NADH-quinone oxidoreductase subunit A	MPAL_v1_550336 *
NADH-quinone oxidoreductase subunit B	MPAL_v1_550337 *
NADH-quinone oxidoreductase subunit C	MPAL_v1_550338 *
NADH-quinone oxidoreductase subunit D	MPAL_v1_550339 *
NADH-quinone oxidoreductase subunit E	MPAL_v1_550340 *
NADH-quinone oxidoreductase subunit F	MPAL_v1_550341 *
NADH-quinone oxidoreductase subunit G	MPAL_v1_550342 *
NADH-quinone oxidoreductase subunit H	MPAL_v1_550343 *
NADH-quinone oxidoreductase subunit I	MPAL_v1_550344 *
NADH-quinone oxidoreductase subunit J	MPAL_v1_550345 *
NADH-quinone oxidoreductase subunit K	MPAL_v1_550346
NADH-quinone oxidoreductase subunit L	MPAL_v1_550347 *
NADH-quinone oxidoreductase subunit M	MPAL_v1_550348
NADH-quinone oxidoreductase subunit N	MPAL_v1_550349 *
ATP synthase subunit	MPAL_v1_460547 *
ATP synthase subunit	MPAL_v1_460548 *
ATP synthase subunit	MPAL_v1_460549 *
ATP synthase subunit	MPAL_v1_460550 *
ATP synthase subunit	MPAL_v1_460551
ATP synthase subunit	MPAL_v1_570110 *
ATP synthase subunit	MPAL_v1_570111 *
ATP synthase subunit	MPAL_v1_570112 *
ATP synthase subunit	MPAL_v1_570113 *
ATP synthase subunit	MPAL_v1_570114 *
S-(hydroxymethyl)glutathione dehydrogenase	MPAL_v1_570123
S-(hydroxymethyl)glutathione dehydrogenase	MPAL_v1_620021
S-formylglutathione hydrolase	MPAL_v1_570124

High   Atm

1109

1110 Supplementary Figure 9. Comparative proteomics of *M. palsarum* NE2 exposed to 1000 p.p.m.v. CH$_4$ (High) in air and 1.9
1111 p.p.m.v. (Atm) CH$_4$ in air (Supplementary table S5). Normalized and standardized expression of enzymes involved in the
1112 central carbon and energy metabolism is shown. Same heatmaps as in Figure 4 and Supplementary Figure 5 but with protein
1113 name and MicroScope accession number. * indicates significant difference in expression between treatments (two sided t-
1114 test).

54
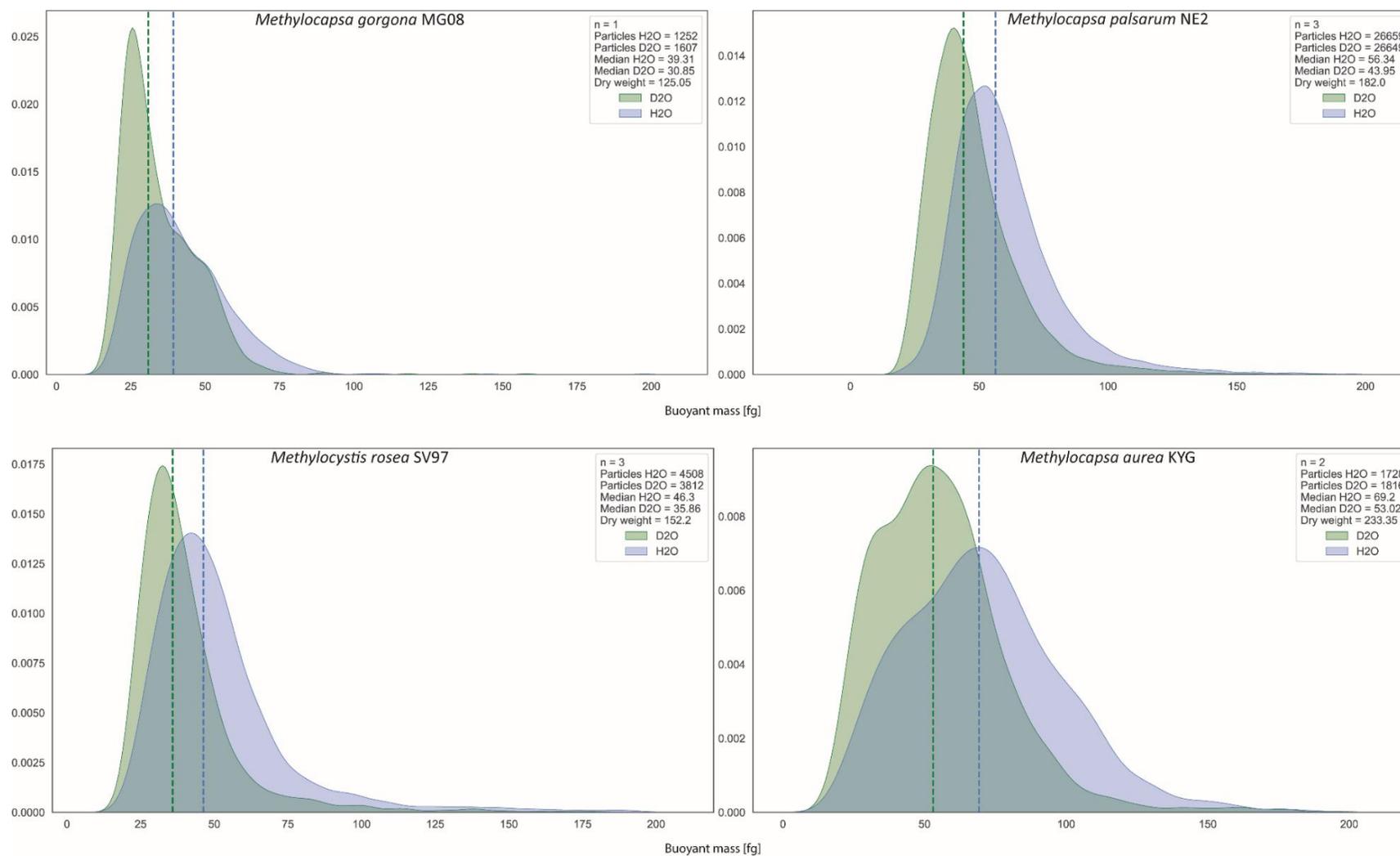
1115

Supplementary Figure 10. Median and buoyant mass distribution of cells of the respective strains in the D_2O-based (green) and H_2O-based (blue) solution. n - number of biological replicates pooled for analysis, Particles H2O - Number of cells measured in H_2O-based solution contributing to the median and distribution, Particles D2O - Number of cells measured in D_2O-based solution contributing to the median and distribution. Median H2O – median of buoyant mass in H_2O-based solution in femtogram, Median D2O – median of buoyant mass in D_2O-based solution in femtogram, Dry weight – estimated cellular dry weight derived from the median of buoyant mass in H_2O-based and D_2O-based solutions in femtogram.
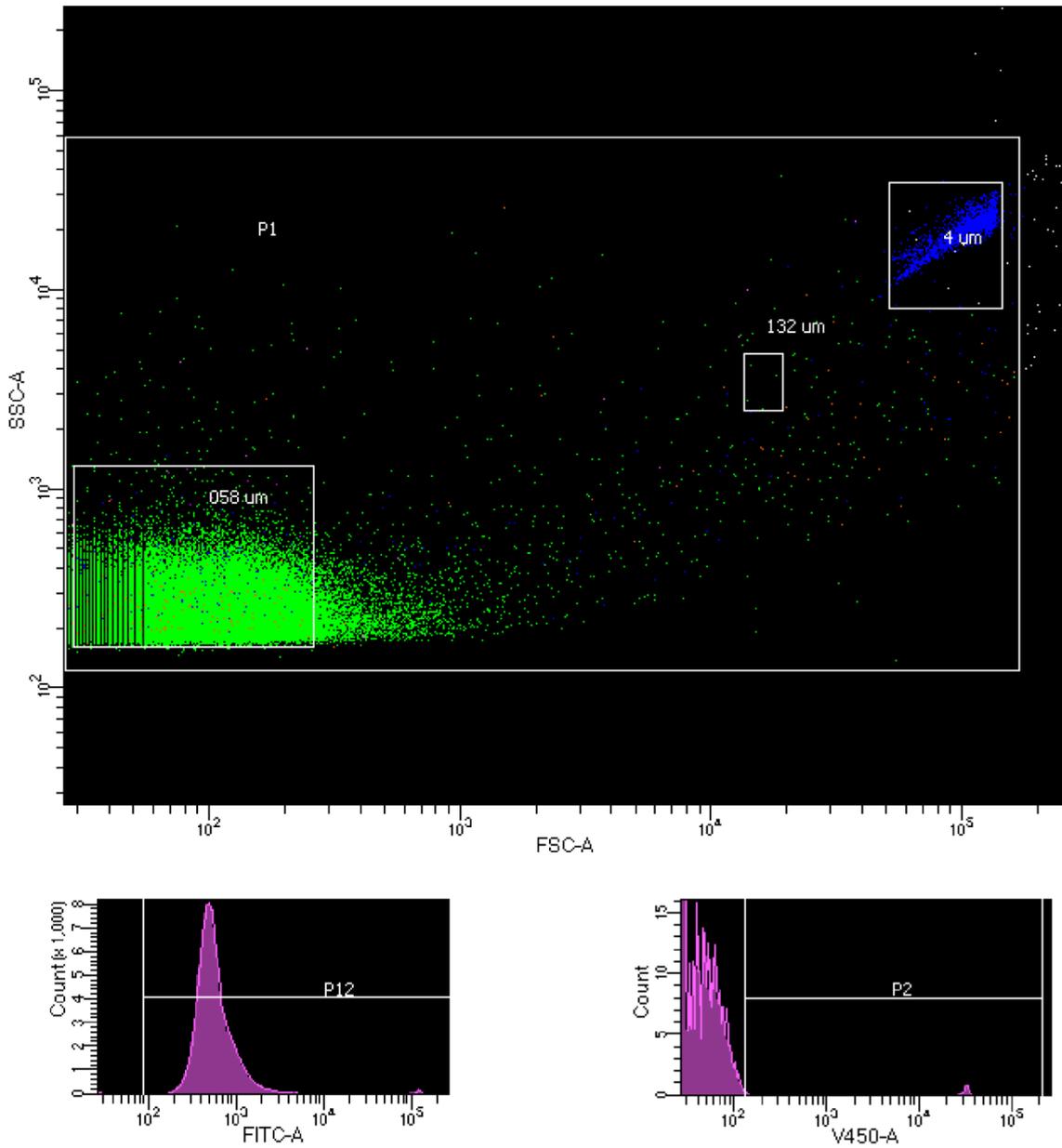
1120

Supplementary Figure 11. Flow cytometry gating strategy example for the cell quantification of filter cultures. SYBR green I stained *M. gorgona* MG08 cells in green. 4 μm counting beads in blue. "058 um" – gate defined using 0.58 μm size beads. "132 um" – gate defined using 1.32 μm size beads. "4 um" - gate defined using 4 μm counting beads. P1 – events of interest for the quantification of cells. SSCA-A – side scatter. FSC- A – forward scatter. FITC-A – signal intensity of events detected in the green channel (*M. gorgona* MG08 cells + counting beads). V450-A – signal intensity of events detected in the blue channel (counting beads).