Bioprocess Development of an Extremophilic Methanotroph and Microalgae Coculture for the Production of a Novel Proteinaceous Biofeed

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Abstract

Industrial methane (CH_4) and carbon dioxide (CO_2) emissions are potent greenhouse gases, and their abatement has become the focus of global warming control strategies. Aerobic methane oxidizing bacteria (methanotrophs), offer the potential to biologically convert CH_4 emissions into single cell protein (SCP) feed for cattle, fish, or poultry. The oxygen requirement for methanotroph activity, however, presents a safety challenge, as methane-oxygen mixtures become explosive between 5% and 15% CH_4 by volume in air. A potential solution to this problem could be to coculture methanotrophs with oxygenic photoautotrophic microalgae in a dilute O_2 environment. The objective of this thesis was to demonstrate proof-of-concept for a novel biotechnological platform with the capability to convert low value CH_4/CO_2 gas waste streams into supplementary biofeedstocks by using a coculture of thermoacidophilic methanotrophic bacteria and photoautotrophic microalgae.

Cocultures of the methanotroph, *Methylacidiphilum* sp. RTK17.1, and the microalga, *Galdieria* sp. RTK37.1, were conducted in batch and continuous systems to determine their performance and stability. Coculture performance was compared to corresponding axenic cultures, and the nutritional suitability of resultant biomass as single cell protein feedstock was assessed. Stable coculture was achieved in both batch and chemostat configurations. In batch, presence of *Galdieria* sp. RTK37.1 significantly enhanced growth (29 %) and methane oxidation (300 %) rates of *Methylacidiphilum* sp. RTK17.1 (*p*-values < 0.05 and < 0.001 respectively), and complete methane removal was achieved without O_2 or air supplementation. In chemostat experiments, *Galdieria* decreased net volumetric O_2 consumption by 46% in coculture, but its oxygenic activity was unable to supply *Methylacidiphilum* with the O_2 required for complete CH_4 removal. *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1, and their coculture each displayed similar nutritional profiles, with protein quality comparing favourably to soybean meal and fishmeal feeds used for animals. It was concluded that *Methylacidiphilum* benefited from the presence of *Galdieria* in microaerobic environments; with interspecies O_2 cross-feeding deemed to play a fundamental role in their interactions.

Existing photoautotroph-methanotroph coculture studies have suffered from a lack of rapid methods to quantitatively evaluate coculture dynamics. Therefore, I developed a technique for measuring the relative abundance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in cocultures using a combination of differential sedimentation, optical density, and fluorescence methodologies (DSOF Method). The validity of the DSOF method was tested using artificial non-growing *Methylacidiphilum*-*Galdieria* mixtures across a wide range of defined biomass concentrations (OD_{600}). The validation showed the absolute error of the derived biomass concentration values was negligible (≤ ± 0.1 A.U.) when [*Galdieria*] ≤ 2.0 A.U. and [*Methylacidiphilum*] ≤ 1.5 A.U. These errors increased to ± 0.2 A.U, for 2.0 A.U. < $[Galdieria]_K$ ≤ 3.23 A.U. DSOF method validation in actively growing cocultures showed that the derived *Methylacidiphilum*-*Galdieria* concentrations were consistent with their expected growth behaviour and prior observations. In conclusion, the DSOF method was determined to be an easy and

accurate method to rapidly quantify the relative concentration of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture.

In order to evaluate the influence of proportional abundance on coculture performance, several batch cocultures with variable initial *Methylacidiphilum*:*Galdieria* mass ratios (with and without CH_4) were performed. For cocultures with dilute initial mass ratio (< 0.18 g_{DW} L^{-1}: g_{DW} L^{-1}) CH_4-containing cocultures fixed more net carbon compared to non CH_4-containing cocultures (17 – 28 % increase, *p*-value < 0.05); as the microalgae supplied the O_2 required for methane oxidation. However, for CH_4-containing cocultures with initial *Methylacidphilum:Galdieria* mass ratios ≥ 0.23 there was a significant reduction of growth rates (66 –100 %, *p*-value <0.001) and net carbon fixation (44 – 62 %, *p*-value < 0.001). Under these conditions, the photo pigment intermediate coproporphyrin, was excreted, and *Galdieria* sp. RTK37.1 exhibited dramatic chlorosis. Coproporphyrin excretion and subsequent chlorosis was triggered by O_2 limitation, as neither were observed if O_2 and CH_4 were regularly replenished in coculture. It was concluded that photoautotrophically grown *Galdieria* sp. RTK37.1 requires a minimum concentration of O_2 to enable adequate pigment production, and that *Methylacidiphilum*'s high affinity for O_2 can induce chlorosis in the microalgae.

Batch coculturing complicates the analysis of potential interspecies interactions as conditions change continuously with time. Furthermore, steady state coculturing helps understand interspecies interactions under defined stable environmental conditions. Therefore, interactions between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in low O_2 environments, during steady state continuous coculture were investigated. By changing the O_2 inlet concentration of steady state continuous axenic cultures and cocultures, it was established that O_2 concentration and net uptake/production rates determined the nature of *Methylacidiphilum*-*Galdieria* interaction in cocultures. In chemostat coculture at inlet O_2 < 2.1% (D.O ≤ 0.198 ± 0.003 mg_{O2} L^{-1}) the interaction was inhibitory for *Galdieria*: *Methylacidiphilum* sp. RTK17.1 benefited from *Galdieria* sp. RTK.37.1, while at the same time harming the microalga by inducing O_2-limitation related chlorosis. At inlet O_2 between 2.1-3.0 % (v/v) the relationship became neutral for *Galdieria*: *Methylacidiphilum* exhibited faster growth, presumably due to greater O_2 availability, allowing CH_4 to be oxidized further, without seemingly affecting *Galdieria*. It was concluded that the nature of the interaction between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 is dependent on the dissolved O_2 concentration, and thus, its monitoring and control is of vital importance for successful cocultures.

Collectively, the obtained results indicate that O_2 plays a fundamental role in *Methylacidiphilum*-*Galdieria* interactions. In general, if O_2 is limiting, *Methylacidiphilum* RTK 17.1 benefits from *Galdieria* photosynthetic activity. However, if *Methylacidiphilum* related O_2 consumption exceeds *Galdieria* photosynthetic O_2 production, the methanotroph can harm the microalgae. This O_2 limitation in *Galdieria* causes the chlorophyll and phycocyanin intermediate, coproporphyrinogen III, to be excreted into the media where it is oxidized into coproporphyrin III. This stops pigment synthesis, which eventually results in pigment degradation (chlorosis). This chlorosis was reversible, however, as pigment synthesis

eventually resumed if headspaces were allowed to accumulate O_2. In conclusion, balancing O_2 production/consumption is vital in *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 cocultures.

This research has made significant contributions to the understanding of *Galdieria* spp., photoautotroph-methanotroph cocultures in general, and more specifically to thermoacidophilic cocultures. It provides vital insight into how O_2 concentrations affect *Methylacidiphilum-Galdieria* cocultures. This interspecies dynamic will serve as the foundation for the conceptualization and design of systems to improve coculture performance. Additionally, the DSOF method enables a simple method to rapidly quantify the relative concentration of microalgae and methanotrophs in coculture. This research expands understanding of photoautotroph-methanotroph cocultures and provides a proof-of-principle for thermoacidophilic SCP bioprocesses. These thermoacidophilic cocultures, which were not previously investigated, offer great potential to convert low (or negative) value industrial gas streams into valuable products (e.g. supplementary biofeedstocks).

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List of Abbreviations

μ	Growth rate
A.U.	Absorbance Units
AMB	Methanotrophic bacterium consortium
AOAC	Association of Official Analytical Communities
BPC	Base Peak Chromatogram
d	Diameter
D	Dilution rate
DO	Dissolved oxygen
DSOF	Differential sedimentation, optical density and autofluorescence method
DW	Dry Weight
EAA	Essential Amino Acid
E-C	Experimental-computational method
EIS	Extracted Ion Chromatogram
EPS	Exopolysaccharides
F	Fluorescence
FAO	Food and Agriculture Organization of the United Nations
GC	Gas chromatographer
GM	*Scenedesmus obtusiusculus*
HPLC	High performance liquid chromatography
IAA	Indispensable Amino Acids
l	Length
LC-MS	Liquid chromatography–mass spectrometry
LDL	Lower detection limit
MDH	methanol dehydrogenase
MMO	methane monooxygenase enzyme
OD	Optical density
OPA	Orthophthaldialdehyde
PHB	Polyhydroxybutyrate
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
R1	Reactor 1 (Axenic *Methylacidiphilum* sp. RTK17.1)
R2	Reactor 2 (Coculture *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1)
R3	Reactor 3 (Axenic *Galdieria* sp. RTK37.1)
rpm	Revolutions per minute
r.t.	Retention Time
SCP	Single Cell Protein
STR	Stirred Tank Reactor
T	Temperature
TCA	Tricarboxylic acid cycle
UNU	United Nations University
WHO	World Health Organization
Subscripts	
D	Derived
K	Known
1	Before centrifugation
2	After centrifugation

Chapter 1. Literature review

1.1 Introduction

Methane (CH_4) is the most important greenhouse gas after carbon dioxide (CO_2) (Cantera, Munoz, *et al.*, 2018). While carbon dioxide abatement has been the main focus of global warming control strategies, methane emission control has been proposed as an important additional strategy (Hamer, 2010). Aerobic methane oxidizing bacteria (methanotrophs), offer the potential to use the methane present in various industrial waste gas streams for growth, with the resulting biomass used as a single cell protein (SCP) feed for cattle, fish, or poultry. Natural gas has been successfully used as a feedstock for SCP production, as feed for the farmed salmon market (Hamer, 2010). Methane oxidation by methanotrophs is an aerobic process, thereby presenting a safety challenge; as methane-oxygen mixtures become explosive between 5% and 15% CH_4 by volume in air (Fei *et al.*, 2014). A potential solution to this problem could be to coculture methanotrophs with oxygen producing photoautotrophic microalgae; as oxygen could be produced and consumed *in-situ*, thus avoiding the development of an explosive gas mixture.

Most microalgae grow well under volumetric CO_2 concentrations from 1 to 5% in the gas phase (Ota, *et al*., 2015), but concentrations greater than 10% may be toxic to some species (Cheng *et al.*, 2016). Flue gases contain CO_2 at typical concentrations between 10-12%, and may contain up to 2400 ppm SO_2, even with efficient scrubbing technologies (Varshney *et al*., 2015). A key challenge for the mass culturing of microalgae is to identify strains that grow well under industrially relevant conditions. These conditions include hot temperatures (due to the bubbling of hot flue gases), concentrated CO_2, and acidic pH (As CO_2 and SO_2 present in flue gases acidify the media) (Zhao & Su, 2014). Few algae are viable at these temperatures and CO_2 concentrations. However, extremophilic microorganisms (including methanotrophs and microalgae) are commonly found in geothermal environments that share physicochemical characteristics with industrial processes (Donachie *et al.*, 2002; Op den Camp *et al.*, 2018). Thus, a coculture of extremophilic methanotrophs and algae could potentially be used to convert waste industrial gas streams into single cell protein for animal feed.

Methanotrophs are bacteria capable of using methane as their sole source of carbon and energy (Ge *et al*, 2014). Most studied methanotrophs are members of the gamma- or alphaproteobacterial classes and are classified as Type I or Type II respectively (Op den Camp *et al.*, 2018). Most described Type I and II methanotrophs are neutrophilic and mesophilic, with the most acidic previously reported pH values, that supported methanotrophic growth, between 4.2 and 5.0 in bacteria isolated from peat bogs (Pol *et al.*, 2007). Evidence of methane oxidation in fumaroles, with temperatures as hot as 70 °C and pH as acidic as 1.8 (Pol *et al.*, 2007), led to the discovery of thermoacidophilic aerobic methane-oxidizing bacteria within the phylum Verrucomicrobiota (formerly Verrucomicrobia) (Whitman *et al.*, 2018) by three independent studies (Dunfield *et al.*, 2007; Islam *et al.*, 2008; Pol *et al.*, 2007). Each of

the identified members from these studies have since been phylogenetically clustered into a single genus, *Methylacidiphilum (*Op den Camp *et al., 2018)*. Mesophilic acidophilic verrucomicrobial methanotrophs have more recently been isolated from cooler geothermal sites and clustered under the genus *Methylacidimicrobium* (van Teeseling *et al.*, 2014). Verrucomicrobial methanotrophs have been identified in geothermal environments over a broad temperature range (23 – 80 °C), but only in acidic conditions (pH 0.8 - 5.0). For the thermoacidophilic verrucomicrobial methanotrophs (*Methylacidiphilum* spp.) the reported optimum temperature for growth is 55 - 60 °C and the optimum pH is 2.0 - 3.5 (Op den Camp *et al.*, 2009; Sharp, Brady, *et al.*, 2014).

Galdieria spp. are a group of extremophilic unicellular red algae which display moderate thermophily (growth up to 56 °C), are tolerant to 100 % CO_2 concentration in the headspace (Varshney *et al.*, 2015), and grow optimally in acidic pH (pH 0 – 4) (Vítová *et al*., 2016). These extremophile algae can grow photoautotrophically, chemoheterotrophically, or mixotrophically (Vítová *et al.*, 2016). The species *Galdieria sulphuraria* can metabolize more than 27 different sugars, including disaccharides, hexoses, pentoses, amino acids, TCA cycle intermediates, and some organic acids (Gaignard *et al.*, 2019). A screening of 42 strains has previously shown biomass is rich in proteins (26-32 % content) and polysaccharides (63 – 69 % content) and poor in lipids (11-18 %) when grown both auto- and heterotrophically (Vítová *et al.*, 2016). In addition, *Galdieria* spp. can accumulate up to 50 % dry cell weight in phycocyanin throughout all growth phases, and can grow to very high cell densities, up to 120 g L^{-1} (Martinez-Garcia, Kormpa, & van der Maarel, 2017).

The overlapping culture conditions of *Galdieria* spp. and *Methylacidiphilum* spp. suggest they could potentially be cocultured for SCP production, using industrial waste gases as substrate. Thus, this literature review focuses on the existing research pertaining to the culture conditions that could affect either *Methylacidiphilum* spp. or *Galdieria* spp. growth in coculture, the interspecies interactions of photoautotroph-methanotroph cocultures, and the production of SCP using methanotroph, photoautotrophs, and cocultures.

1.2 Verrucomicrobial methanotrophs

Verrucomicrobial methanotrophs were first reported by three independent studies in 2007 and 2008 (Dunfield *et al.*, 2007; Islam *et al.*, 2008; Pol *et al.*, 2007). Since then, most of the studies have focused on the ecology (Op den Camp *et al.*, 2009; Sharp *et al.*, 2014; van Teeseling *et al.*, 2014), genomics (Anvar *et al.*, 2014; Hou *et al.*, 2008; Khadem *et al.*, 2012; Kruse *et al.*, 2019), metabolism (Carere *et al.*, 2017; Khadem *et al.*, 2012) and physiology (Carere *et al.*, 2021; Carere *et al.*, 2019) of the thermoacidophilic methanotrophs within this phylum (Anvar *et al.*, 2014). Despite the prevalence of work over the past 20 years investigating the usage of conventional (Type I and Type II) methanotrophs for the production of biofuels, ectoines, biopolymers, methanol and exopolysaccharides (Cantera *et al.*,

2019; Strong *et al.*, 2016; Strong, Xie, & Clarke, 2015) no studies have explicitly reported on the potential industrial applications of verrucomicrobial methanotrophs.

The Rotokawa region in Aotearoa New Zealand is a geothermal area with acidic soils and high CH_4 and CO_2 emissions (Sharp, Smirnova, *et al.*, 2014), which make it a likely candidate to find verrucomicrobial methanotrophs. The strain *Methylacidiphilum* sp. RTK17.1 was isolated in this region. It grows optimally at pH 2.5, 50 °C (T_{max} 60 °C), oxidizes CH_4, fixes CO_2, fixes N_2 under nitrogen limiting conditions, accumulates glycogen, and rapidly consumes H_2 under microaerophilic conditions. CO_2 fixation occurs in cultures supplied with H_2, as the sole reductant and O_2 as the sole oxidant (Carere *et al.*, 2017).

1.2.1 Carbon and energy sources

There are several studies on the methane oxidation and carbon fixation of verrucomicrobial methanotrophs, which are summarized in Op den Camp *et al.* (2018). In brief, methanotrophs oxidize methane to methanol using the methane monooxygenase enzyme (MMO), which is further metabolized into formaldehyde using a methanol dehydrogenase (MDH) in proteobacterial methanotrophs (Jiang *et al.*, 2010). The main distinction between Type I or Type II methanotrophs is the pathway used for assimilation of formaldehyde into biomass; the ribulose monophosphate pathway for Type I methanotrophs, and the serine pathway for Type II methanotrophs (Kalyuzhnaya, Puri, & Lidstrom, 2015; Strong *et al.*, 2015). However, all sequenced *Methylacidiphilum* genomes show that MMO is used to convert methane into methanol, but unlike proteobacterial methanotrophs, in *M. fumariolicum* SolV, a methanol dehydrogenase (MDH) oxidizes methanol directly to formate without production of the formaldehyde toxic intermediate. Pol *et al.* (2014) showed that this enzyme is dependent on lanthanides at nanomolar concentrations, thus growth depends on rare earth metals presence. Lanthanum and cerium have been found to stimulate growth the most for strain *M. infernorum* V4 (Sharp, Smirnova, *et al.*, 2014). Verrucomicrobial methanotrophs oxidise methane terminally to CO_2, which they then assimilate using the Calvin-Benson-Bassham cycle (Hou *et al.*, 2008; Khadem *et al.*, 2011). Khadem *et al.* (2011) reported no batch growth on methane when CO_2 concentrations were < 0.3 % (v/v) in the headspace, and maximum growth rate on CO_2 at 1% (v/v). The highest CO_2 concentration at which *Methylacidiphilum* has been grown is 92.7% in chemostat (Khadem, van Teeseling, *et al.*, 2012).

In soil profiles in the Rotokawa geothermal field, Verrucomicrobial hydrogenase and methane monooxygenases genes were detected in soils associated with dilute CH_4 and H_2 soil gas concentrations. As CH_4 and H_2 concentrations increased, the relative abundance of *Methylacidiphilum* sharply decreased (Carere *et al.*, 2017). It is possible then that *Methylacidiphilum* strains might scavenge methane at low concentrations and might be able to remove it from low concentration flue gases. *Methylacidiphilum* strains have been grown at CH_4 headspace concentrations as low as 2 % (Pol *et al.*, 2007) and 2.3 % (Khadem *et al.*, 2011) for batch and chemostat operation respectively. Op den Camp *et al.* (2018) suggested the use of verrucomicrobial methanotrophs in biofiltration of methane, but there is no published study regarding this matter. Conversely, the absence of Verrucomicrobiota at

soil depths with high CH_4 concentrations might indicate an inhibiting effect, but there is reported growth at 60 % CH_4 (v/v) in batch (Mohammadi *et al.*, 2017) and at 50 % CH_4 (v/v) in chemostat (Khadem *et al.*, 2012). Clearly, more research is needed on the effect of methane concentration on *Methylacidiphilum* spp. growth. In this regard, chemostat experiments at different methane concentrations might help elucidate this effect.

Methylacidiphilum spp. can use other sources of carbon and energy other than CH_4, Khadem *et al.* (2012) showed that the genes encoding the enzymes required for the TCA cycle were expressed for exponentially growing cells without nutrient limitation, and for cells grown on chemostat with either oxygen or nitrogen limitation. This suggests that growth on two carbon compounds could be possible. However, Pol *et al.* (2007) found that for *Methylacidiphilum* SolV a 1 g L^{-1} concentration of either acetate, malate, succinate, formate, formaldehyde, or yeast extract inhibited batch growth on methane, at pH 2. Interestingly, formate at pH 5 did not inhibit the bacterium. Growth on methanol was achieved, but it completely repressed methane consumption. The authors did not specify if growth in these substrates in the absence of methane was attempted, however Islam *et al.* (2008) observed no growth for isolate *Methylacidiphilum* Kam1 at pH 3.5 on ethanol, acetate or glucose in the absence of methane. This isolate was able to grow on methanol in concentrations from 1 to 36 µM. Similar results were obtained by Dunfield *et al.* (2007), who observed growth for isolate V4 on methane and methanol, but not on multicarbon substrates. Dunfield *et al.* (2007) also observed that the maximum growth rate on methanol depended on the presence of CO_2 in the headspace. Picone *et al.* (2020) recently reported growth of SolV on ethane and propane, but not on butane. The presence of the TCA cycle suggests that it might be possible that some organic acids act as sources of energy at low concentrations (Op den Camp *et al.*, 2009), and Carere *et al.* (2021) reported that *Methylacidiphilum* sp. RTK17.1 can use formic acid as a substrate in chemostat growth if the cells are able to maintain pH homeostasis. To achieve this, the methanotroph was first grown in chemostat using methanol as a substrate and when steady state was achieved, the inlet substrate was switched to formic acid (Carere *et al.*, 2021). *Methylacidiphilum* spp. metabolic flexibility suggests the possibility of it using metabolites produced by other species in coculture, however there are no reports of coculture growth of verrucomicrobial methanotrophs.

1.2.2 Nitrogen metabolism

Methylacidiphilum spp. can readily assimilate ammonium (NH_4^+) or nitrate (NO_3) as a nitrogen source (Islam *et al.*, 2008; Khadem *et al.*, 2012; Pol *et al.*, 2007), it can also fix dinitrogen gas (N_2) under microaerophilic conditions (Carere *et al.*, 2019; Khadem *et al.*, 2010). Strain *M*. SolV showed a maximum growth rate of 0.07 h^{-1} when grown with ammonium and 0.04 h^{-1} when grown on nitrate, both cases in batch culture at 55 °C with no limitation. The slower growth on nitrate is attributed to the additional reducing energy required to reduce nitrate to ammonium (Khadem, van Teeseling, *et al.*, 2012).

High concentrations of ammonium can be problematic for *Methylacidiphilum* spp. Ammonia (NH_3) has a comparable structure to CH_4 and is readily oxidized by the MMO enzyme to produce the highly toxic hydroxylamine (NH_2OH) (Mohammadi *et al.*, 2017). NH_2OH needs to be quickly detoxified, and this is achieved by using a hydroxylamine dehydrogenase enzyme to convert it back to NH_4^+ or NO_2^- (Mohammadi *et al.*, 2017). Nitrite is also toxic and is further converted by denitrification enzymes to toxic intermediate nitric oxide (NO) and then to nitrous oxide (Mohammadi *et al.*, 2017). In their natural acidic environment *Methylacidiphilum* SolV is subjected to ammonium concentrations ranging from 1 to 28 mM (Khadem *et al.*, 2010), Mohammadi *et al.* (2017) suggested that the real substrate for pMMO to produce nitrite is NH_3, and not NH_4^+, and it would follow that decreasing the culture's pH should limit NO_2^- production. In continuous cultures under O_2 limitation, 0.3 µM of CH_4 in the liquid, and 4 mM NH_4^+, NO_2^- production was not detected at pH < 4.0. At pH 5.5, NO_2^- concentration was 420 µM in the reactor and its production rate around 48 nmol NO_2^- mg_{DW}^{-1} h^{-1}. Mohammadi *et al.* (2017) also observed that increasing the headspace CH_4 concentration from 0.5 to 8 % (v/v) increases the ammonia oxidation affinity constants from 1.25 to 70 mM for NH_4^+, and from 4.9 to 272.3 µM for NH_3, thus ammonia oxidation is sensitive to methane concentrations.

Khadem *et al.* (2010) observed that N_2 fixation occurs only when the availability of nitrogen compounds becomes limiting, and then only when the oxygen concentration in the headspace is < 2 % v/v. Nitrogen fixing cultures with O_2 concentration in the headspace from one to two % v/v grew linearly, and growth accelerated at concentrations < 1 % v/v. Maximum nitrogenase activity for *Methylacidiphilum* SolV was found at 0.5 % v/v (Khadem *et al.*, 2010). For chemostat operation, growth on N_2 was only observed on pO_2 values < 0.5 % saturation (Khadem *et al.*, 2012). Khadem *et al.* (2012) also reported that cells grown with ammonia to the stationary phase, when transferred to a medium with N_2 as the sole source of nitrogen, took 13 days to grow. The lag phase for similar cells transferred to an ammonium containing medium was one day, so the lag phase was not a response to inactivation of metabolism. When N_2 fixing cultures were transferred to fresh nitrogen free medium, the lag phase was also one day. The long adaptation was credited to the induction of the nitrogenase enzyme, which would rely on recycling other proteins or stored nitrogen for its biosynthesis. This is consistent with the upregulation of several genes related to nitrogenase biosynthesis and transcriptional regulation reported by Carere *et al.* (2019) in response to N_2 fixing conditions for *Methylacidiphilum* sp. RTK17.1 grown in chemostats.

Reported maximum growth with N_2 as nitrogen source is slower (0.025 h^{-1}) than with either nitrate or ammonium (Khadem, Pol, *et al.*, 2012), which is expected as nitrogen fixation is an endergonic process. Additionally, N_2 fixing cells present a lesser biomass yield on CH_4, (Carere *et al.*, 2019; Khadem *et al.*, 2010), a slightly higher O_2 consumption, and a higher production of CO_2 when compared to ammonium grown cells (Khadem *et al.*, 2010). For *Methylacidiphilum* sp. RTK17.1 nitrogen fixing in the absence of a complimentary source of respiratory energy, resulted in an increased glycogen accumulation and a decreased protein concentration (Carere *et al.*, 2019).

1.2.3 Glycogen accumulation

Several methanotrophs can store carbon in the form of glycogen (Gilman *et al.*, 2015), polyhydroxybutyrate (PHB) (Rostkowski, Pfluger, & Criddle, 2013), or produce exopolysaccharides (EPS) (Cantera, Munoz, *et al.*, 2018) when exposed to a critical nutrient limitation or in a carbon rich environment (Carere *et al.*, 2019). While genes encoding for PHB production are missing from analyzed *Methylacidiphilum* genomes (Khadem, van Teeseling, *et al.*, 2012) and there are no reports on EPS production; there is documented glycogen synthesis in *Methylacidiphilum fumarolicum* Sol IV (Khadem, van Teeseling, *et al.*, 2012) and *Methylacidiphilum* sp. RTK17.1 (Carere *et al.*, 2019).

Khadem, van Teeseling, *et al.* (2012) showed that *M*. *fumarioculum* SolV accumulates glycogen in response to nitrogen limitation. The methanotroph was cultivated in a bioreactor with methane excess and ammonium as a nitrogen source. Dissolved oxygen concentration was maintained > 2 % to avoid nitrogen fixation. The culture grew exponentially until ammonium was depleted, which was accompanied by a sharp increase in dissolved oxygen concentration and a gradual decrease in growth rate. A small amount of glycogen may have been synthesised during exponential growth, as it accounted for 2 % in weight of dry biomass at the end of this phase. After ammonium depletion, glycogen accumulated in two distinct phases. During the first phase (which lasted 1.5 days) cell numbers, total carbon, dry weight, and optical density doubled. Glycogen content increased to 26% of dry weight, total carbon content per cell more than doubled. The authors claimed that the required proteins for cell growth came at the expense of proteins and nitrogen from the supernatant, as total protein content of the culture remained constant. During the second phase, optical density and dry weight slowly increased for seven more days; but cell numbers remained roughly constant. Glycogen in the cells -which accounted for 36 % of dry weight at the end of this phase- apparently causes increased light scattering.

Carere *et al.* (2019) expanded upon the factors that induce glycogen accumulation in *Methylacidiphilum* sp. RTK17.1 grown in chemostats. The effect of oxygen (57.5 % or 0.17 % O_2 saturation), nitrogen source (NH_4^+ or N_2), and hydrogen supply (0.4 % or 0 % v/v) were tested. When grown under O_2-replete conditions, in the presence of H_2 and NH_4^+, biomass and glycogen productivity were 8.32 and 0.94 mg L^{-1} h^{-1} respectively, and glycogen content was 11.26 % w/w of the dry biomass. This glycogen content contrasts with the 2 % obtained at the end of exponential growth by Khadem, van Teeseling, *et al.* (2012), but the differences in growth conditions do not allow to make comparisons between strains. Switching to oxygen limitation decreased biomass growth rate by 33.1 %, and increased glycogen content to 20.23 %. Removing NH_4^+ from the media - thus triggering nitrogen fixation- further decreased biomass productivity (by 8.61%), but glycogen content remained approximately the same (20.00 %). Finally, removing H_2 from the headspace under nitrogen fixing conditions further decreased biomass productivity (by 18.67%) and significantly increased glycogen content to 48.86 % w/w. This corresponded to a 2.03-fold increase in glycogen volumetric productivity when compared to nitrogen fixing conditions with hydrogen supply. Apparently, conditions where energy availability is constraining

cell growth increase intracellular glycogen production. The authors speculate that this is an energy saving technique, as protein synthesis require approximately 19-fold more ATP than saccharide polymerization. Thus, preferentially allocating resources towards glycogen synthesis over growth can benefit cell survivability during periods of starvation. Hydrogen oxidation serves to counteract the energetic demand of N_2 fixation and thus influences the production of glycogen (Carere *et al.*, 2019).

To assess the physiological role of glycogen Khadem, van Teeseling, *et al.* (2012) grew cultures with ^{13}C labeled carbon dioxide and methane until the second phase of glycogen accumulation to store ^{13}C labeled glycogen. When cells were transferred to a medium without methane, production of $^{13}CO_2$ indicated catabolism of glycogen. The production of $^{13}CO_2$ continued for 47 days. A control with cells that accumulated glycogen to the end of exponential growth produced $^{13}CO_2$ for only 3 days. Cells that accumulated glycogen, when transferred to a methane-free medium with 2mM of ammonium, displayed no increase to cell numbers and a decreased optical density (OD_{600}) over 10 consecutive days. This observed decrease in optical density was attributed to glycogen consumption, as ^{13}C labeling suggested it was depleted after the starvation period. In media with methane, but no ammonium, the optical density remained stable. The evidence suggests that glycogen can be consumed to meet energy demands but it cannot sustain cell division in *Methylacidiphilum* (Khadem, van Teeseling, *et al.*, 2012).

The role of glycogen on the viability of cells during a 70-day methane starvation, was also studied by Khadem, van Teeseling, *et al.* (2012). Viability was measured by the duration of the lag phase after inoculation with optimal growth conditions. Cells grown until the end of the exponential phase rapidly lost viability after only 4 days of starvation and decreased 4.5 times in number after the 70-day incubation period. In contrast, glycogen-laden cultures decreased in number 2.4 times and showed an unchanged 28 h lag phase for ~40 days off methane starvation. Lag phase sharply increased after 45 days, presumably due to glycogen depletion, which was 47 days for similarly grown ^{13}C labeled cells. Collectively these results indicate glycogen is used by *Methylacidiphilum* spp.to retain viability after periods of starvation.

1.3 Thermoacidophilic microalgae

Galdieria spp. are unicellular thermoacidophilic red algae (Gross & Schnarrenberger, 1995), members of the Cyanidiophyceae (Sinetova, Markelova, & Los, 2006), which can typically grow autotrophically, heterotrophically, and mixotrophically (Schmidt, Wiebe, & Eriksen, 2005). They favour growth in cryptoendolithic habitats, which are usually challenging environments for photosynthetic organisms due to restricted access to light. Part of their success comes from the tolerance to high concentrations of salt and heavy metals (López *et al.*, 2019). Most of the research on *Galdieria* spp. is centred around heterotrophic and mixotrophic growth of *Galdieria sulphuraria* for production of phycocyanin (Graverholt & Eriksen, 2007; Moon *et al.*, 2014; Sloth, Wiebe, & Eriksen, 2006), floridoside (Martinez-Garcia & van der Maarel, 2016), and glycogen (Martinez-Garcia *et al.*, 2017; Martinez-Garcia, Stuart, & van der

Maarel, 2016), and for recovery of nutrients from urban wastewaters (Selvaratnam *et al.*, 2014), rare earth elements (Minoda *et al.*, 2015) and copper (Ostroumov, Shestakova, & Tropin, 2015).

Galdieria spp. are often found in hostile environments like volcanic hot sulphur springs and solfatara soils. It can grow in temperatures up to 56 °C, and pH as acidic as 0.0 (Marquardt & Rhiel, 1997; Vítová *et al.*, 2016). Optimal growth conditions for *G*. *sulphuraria* are temperatures between 40-45 °C and at pH 1-2 (Schmidt *et al.*, 2005). It has been found to be tolerant of toxic metal ions (Al^{3+}, Cd^{2+}, Hg^{+}, and Hg^{2+}) (Thangaraj *et al.*, 2011) and high salt concentrations (Schönknecht *et al.*, 2013). *Galdieria sulphuraria* typical cell diameters are ~ 3 - 9 µm (Vítová *et al.*, 2016). Photoautotrophically grown cells are usually oval-shaped and have an average size of 3.8 x 5.0 µm (Sinetova *et al.*, 2006; Stadnichuk *et al.*, 1998), whereas heterotrophically grown cells are typically 30% larger (Barone *et al.*, 2020).

A distinguishing characteristic of *Galdieria spp.* is its capacity to grow heterotrophically on a variety of reduced carbon substrates. Heterotrophic growth has been confirmed on 27 different substrates, including disaccharides, hexoses, pentoses, amino acids, TCA cycle intermediates, and some organic acids (Gross & Schnarrenberger, 1995; Vítová *et al.*, 2016). Lag phases of two to three days are typically observed before heterotrophic (Gross & Schnarrenberger, 1995) or autotrophic growth (Sinetova *et al.*, 2006). Reported maximum growth rates vary between 0.10-0.35 day^{-1} for autotrophic growth, and 0.23-1.2 day^{-1} for mixotrophic and heterotrophic growth (Barone *et al.*, 2020; Mozaffari *et al.*, 2019; Sakurai *et al.*, 2016; Sloth *et al.*, 2006). Trophic lifestyle is also known to influence biomass compositions of *Galdieria spp*.; mixotrophic growth conditions typically report the greatest biomass concentrations (Sakurai *et al.*, 2016), autotrophic growth is associated with an accumulation of fatty acid methyl esters, phytol (an acyclic diterpenoid alcohol constituent of chlorophyll), and heterotrophic cells displaying elevated contents of polysaccharides, sugars, ergosterol, stearic acid, and oleic acid (Barone *et al.*, 2020).

1.3.1 Pigments and photoinhibition

The pigment apparatus produced by *Galdieria* spp. is similar to photosynthetic cyanobacteria (Sinetova *et al.*, 2006); their main pigments being chlorophylls, phycobiliproteins (phycocyanin), and carotenoids (Marquardt & Rhiel, 1997; Sloth *et al.*, 2006; Stadnichuk *et al.*, 1998). Some strains of *Galdieria sulphuraria* develop chlorophyll and phycocyanin when growing heterotrophically in the dark, while others are reported to lose these pigments (Gross & Schnarrenberger, 1995).

Thangaraj *et al.* (2011) reported that laboratory cultures of *Galdieria* spp. can be inhibited by light intensities > 40 µmol photons m^{-2} s^{-1} as its photosynthetic systems are adapted to the low light conditions typical of endolithic habitats where this microalga thrives, and *Galdieria sulphuraria* lacks the gene pivotal for the ability to adapt to changing light conditions**.** However, the microalga has been reported to grow to high biomass concentrations (1.7-5 g_{DW} L^{-1}) under relatively low light intensities (15–35 $\mu mol_{photons}$ m^{-2} s^{-1}) (Sloth *et al.*, 2006). Thangaraj *et al.* (2011) reported mixotrophic growth to be

independent from light for *Galdieria sulphuraria* for light intensities 0 - 117 $\mu mol_{photons}$ m^{-2} s^{-1}, and declining growth rates at light intensities > 198 µmol photons m^{-2} s^{-1}. Oesterhelt *et al.*, (2007) found strong photoinhibition at > 225 µmol photons m^{-2} s^{-1}. Sloth *et al.* (2006) also reported severely reduced phycocyanin contents and growth rates at light intensities > 395 $\mu mol_{photons}$ m^{-2} s^{-1}. Liu *et al.*,(2021) were able to obtain high concentration cultures of mixotrophic and autotrophic Galdieria under high-light stress, along reductions in photosynthetic pigments, which the author claimed indicated a mechanism to relieve photoinhibition effects.

For *Galdieria sulphuraria* low light intensity stimulates pigment synthesis while high intensity represses it (Sloth *et al.*, 2006). For example, Liu *et al.* (2021) reported that changing light intensity from 15 - 25 to 65 - 85 $\mu mol_{photons}$ m^{-2} s^{-1} caused a decrease of 55.4 % in chlorophyll fluorescence and 78.4 % in phycocyanin fluorescence. Phycocyanin degradation could be a photoprotection response to high light exposure (Liu *et al.*, 2021). This could pose a problem for achieving high concentration of *Galdieria* spp. in coculture, as cell shading effects from a partner could rapidly slow down growth at low light intensities, and increasing light intensity could trigger photoinhibition.

1.3.2 N-starvation and chlorosis

In response to nitrogen starvation, *Galdieria* spp. slow rates of cell division, and prioritise the accumulation of nitrogen-free storage compounds (e.g. glycogen) (Martinez-Garcia *et al.*, 2016; Shimonaga *et al.*, 2008) to the detriment of photosynthetic apparatus production (Sinetova *et al.*, 2006). As with *Methylacidiphilum* sp. RTK17.1, there is little glycogen production during exponential growth (autotrophic, mixotrophic, or heterotrophic) (Sakurai *et al.*, 2016). In the stationary phase, however, glycogen accumulation is greatest for mixotrophic growth and least for autotrophic growth (Sakurai *et al.*, 2016).

Upon the onset of N-starvation, *Galdieria sulphuraria* cells transition from their characteristic blue-green colour to brown-green (Salbitani & Carfagna, 2020). These colour changes are associated with changes to their absorption spectrum, as evidenced by the decrease in the chlorophyll absorption peak at 678 nm and the absorption region at 620 nm (Stadnichuk *et al.*, 2011). If N-starvation persists, cells become almost colourless due to pigment degradation. The process of pigment degradation, 'chlorosis' (Salbitani & Carfagna, 2020; Stadnichuk *et al.*, 2011), in *G. sulphuraria* occurs in two stages (Sinetova *et al.*, 2006). In the first stage (~3-4 days) phycobiliproteins degrade completely. Changes in absorption spectra indicate that phycocyanin synthesis and degradation start as soon as one day after starvation starts. Synthesis of chlorophyll and carotenoids also stops in this stage, but the pigments do not degrade. During the second stage of chlorosis, (days 4-14), chlorophylls and carotenoids rapidly degrade to a minimum level, and do not recover (Sinetova *et al.*, 2006).

1.3.3 O_2 Limitation and coproporphyrin excretion

When subjected to low O_2 concentrations, during heterotrophic or mixotrophic growth, some species of *Galdieria* have been found to excrete the tetrapyrrole compound coproporphyrin III (Sarian, Rahman, Schepers, & van der Maarel, 2016; Zhu, Wei, & Pohnert, 2022). This has been reported for both heterotrophic- and mixotrophically grown cultures of *Galdieria partita* (Stadnichuk *et al.*, 1998), *Galdieria sulphuraria* Strain 074G (Sarian *et al.*, 2016), and *Galdieria sulphuraria* UTEX 2919 *(Zhu et al., 2022)* under O_2 limitation.

Coproporphyrin excretion was originally attributed to glucose inhibition of tetrapyrrol biosynthesis (Stadnichuk *et al.*, 1998). However, Sarian *et al.* (2016) more recently cultivated *G*. *sulphuraria* autotrophically, heterotropically (with added air), and mixotrophically (with and without added air), and found coproporphyrin excretion to occur only in anoxic or microoxic conditions. Even though the microalgae did not grow well under the microoxic conditions, coproporphyryn was detected when dissolved O_2 concentration dropped below detection limits. In separate mixotrophic cultures with variable areation, coproporphyryn increased (Sarian *et al.*, 2016). Coproporphyryn was also observed in O_2 limited batch cultures using 1 % w/v of either glucose, galactose, dulcitol, or sucrose as substrates (Sarian *et al.*, 2016). Thus, it was concluded that O_2 limitation (and not glucose) was causing coproporphyrin excretion.

Coproporphyrin is an intermediate in the biosynthesis of both phycocyanin and chlorophyll (Stadnichuk *et al.*, 1998). In the shared part of both pigments' biosynthetic pathway the intermediate coproporphyrinogen III is oxidised by an oxygen-dependent oxidoreductase, HemF, into protoporphyrinogen IX (Sarian *et al.*, 2016; Skotnicová *et al.*, 2018). HemF is completely inhibited by anaerobic condition, so under oxygen limitation coproporphyrinogen III acumulates and is eventually excreted into the media, where the acidic conditions oxidizes it into the red-coloured coproporphyrin III (Sarian *et al.*, 2016). As a consequence, pigment synthesis stops, and this eventually results in loss of pigments and yellowed-coloured microalgae cells (Stadnichuk *et al.*, 1998).

There are currently no reports of coproporphyrin excretion during photoautotrophic growth of *Galdieria* spp. However, Zhu *et al.* (2022) recently reported the added O_2 consumption from bacterial community members, resulted in a substantially increased coproporphyrin excretion in a mixotrophically grown mixed-culture, consisting of *Galdieria sulphuraria* UTEX 2919 and a bacterial community derived from industrial wastewater. Slower growth rates and a lesser biomass titre (in comparison with axenic *Galdieria sulphuraria* UTEX 2919) was also observed (Zhu *et al.*, 2022). Thus, it is then possible that a coculture partner could trigger coproporphyrin excretion in autotrophic *Galdieria spp.* via excessive O_2 consumption.

1.4 Methanotroph-photoautotroph cocultures

Methanotroph-photoautotroph cocultures have received increasing attention recently because of their potential to simultaneously convert low- or negative value waste gas emissions (e.g. CH_4, CO_2) into a variety of products. The majority of these studies have investigated bioprocesses using non-extremophilic proteobacterial methanotroph and photoautotrophic strains. Cocultures of the methanotroph *Methylomicrobium alcaliphilum* 20z and the cyanobacterium *Synechococcus* PCC 7701 have been shown to successfully utilize biogas (58 % CH_4, 42 % CO_2, 0.3 % H_2S) and synthetic natural gas (80 % CH_4, 3 % CO_2, 17 % N_2) as feedstocks without the need for O_2 supplementation (Hill, Chrisler, Beliaev, & Bernstein, 2017). In illuminated batch cocultures performed alongside axenic controls, the axenic *Synechococcus* displayed the fastest growth rate and greatest biomass concentrations. Conversely, the coculture had higher biomass concentrations but slightly lesser specific growth rates compared to the axenic *Methylomicrobium* controls. Increasing CO_2 supplementation inhibited growth performance of all cultures compared to biogas or other higher CH_4 gas concentrations. Cocultures also ultimately exhibited light limitation. In illuminated chemostat cocultivations using synthetic natural gas, sufficient O_2 was produced by the cyanobacterium to support *Methylomicrobium* growth without elevating dissolved O_2 concentrations. These results suggest continuous photoautotroph-methanotroph cocultivations can proceed in the absence of explosive CH_4/O_2 mixtures (Hill *et al.*, 2017).

Rasouli *et al* (2018) studied the conversion of nutrients from a potato processing plant wastewater stream (3000 mg L_{COD}^{-1}, 19 mg_N L^{-1} ammonium, 14 mg L^{-1} phosphorus) into single cell protein using a consortium of the methanotroph *Methylococcus capsulatus* and the microalgae *Chlorella sorokiniana*. Batch experiments demonstrated that interspecies interactions could affect biomass productivity and composition; as cocultures exhibited a slower growth rate, decreased protein content, and increased fat and carbohydrate contents compared to axenic control cultures (Rasouli, Valverde-Pérez, D'Este, De Francisci, & Angelidaki, 2018). A study from van der Ha *et al* (2011) used unspecified methane oxidizing and microalgae communities to investigate methanotroph-photoautotroph syntrophic interactions. In a series of batch coculture experiments it was found that the coculture yielded 1.6-fold more biomass than the control without microalgae, and methane oxidation rates were not significantly decreased by the microalgae. Moreover, it was found that the coculture tended to grow forming flocs where around half the algal and bacterial biomass accumulated, which made separation from the culture easier. The authors theorized that the flocs formed due to a mutually beneficial syntrophic relationship (van der Ha, Bundervoet, Verstraete, & Boon, 2011).

Methanotrophs have also been benefited by coculture with other heterotrophic bacterial strains. The bacterium *Sphingopyxis* sp. NM1 has been shown to stimulate growth of the methanotroph *Methylocystis* sp. M6 in a concentration dependant manner (Jeong & Kim, 2018). Iguchi, Yurimoto, and Sakai (2011) reported that the cell masses of both the bacteria *Rhizobium* sp. Rb122 and the methanotroph *Methylovulum miyakonse* HT12 increased when cocultured in batch, with faster growth for the methanotroph. This effect was also observed when *M. miyakonse* was grown using small

amounts of *Rhizobium* sterilized spent medium. Cobalamin (Vitamin B12) was found to be the growth stimulating factor. Other methanotrophs tested showed a growth stimulating effect of cobalamin only if they did not produce it themselves (Iguchi *et al.*, 2011). The methylotroph *Hypomicrobium* sp. has been shown to enhance methane oxidation rates of *Methylocystis* sp. in cocultures. While CH_4 oxidation rates and the biomass concentration of the methanotroph increased with time, *Hypomicrobium* populations tended to decrease. A fed batch bioprocess, in which the methylotroph was contained within a dialysis membrane helped achieve higher methane oxidation rates while maintaining constant biomass concentrations (Jeong & Kim, 2018).

1.4.1 Photoautotroph-methanotroph interactions

Inoculum proportion is a key factor that contributes to mixed growth performance in cocultures, this has been established for photoautotroph:heterotroph cocultures involving microalgae (Santos & Reis, 2014) and has also been observed in methanotroph cocultures involving other heterotrophs. As an example, Jeong, Cho, and Kim (2014) demonstrated that the bacterium *Sphingopyxis* sp. NM1 was able to stimulate growth and CH_4 oxidation rates of *Methylocystis* sp. M6 when using a 1:9 M6:NM1 volumetric ratio; but not when the ratio was 9:1 or 1:1. Presence of *Sphingopyxis* enhanced transcription of genes involved in methane oxidation when it was more abundant than *Methylocystis* (Jeong *et al.*, 2014). A similar concentration-dependant effect on methane oxidation was observed within mixed cultures of *Methylocystis* sp. M6 and *Hyphomicrobium* sp. NM3 (Jeong & Kim, 2018).

For photoautotroph-methanotroph cocultures, specifically under O_2 limiting conditions, increasing photoautotroph:methanotroph ratios tends to hasten coculture growth when coupling methanotrophs with relatively slow-growth photoautotrophs. This has been observed for cocultures of the methanotroph *Methylococcus capsulatus* with the microalga *Chlorella sorokiniana* (Badr, Whelan, He, & Wang, 2020), and for the methanotroph *Methylomicrobium buryatense* 5GB1 with the cyanobacterium *Arthrospira platensis* (Badr, He, & Wang, 2022). In both cases, faster growth was attributed to an increase in O_2 availability due to increased photoautotroph concentration (Badr *et al.*, 2022). This was confirmed for *M*. *buryatense-A*. *platensis* cocultures, as increasing external O_2 supply had an impact on the final photoautotroph:methanotroph ratio, and hence in *M*. *buryatense* concentration (Badr *et al.*, 2022). When O_2 is not limiting, the abundance of photoautotroph does not necessarily benefit the methanotroph in coculture. Badr *et al.* (2020) found, when pairing the methanotroph *Methylomicrobium alcaliphilum* 20ZR with the cyanobacterium *Synechococcus* sp., that the photoautotroph:methanotroph ratio did not affect coculture growth. Since the cyanobacterium grew much faster than the methanotroph, the limiting factor was CH_4 transfer rather than O_2 availability (Badr *et al.*, 2020).

There are also cases of microalgae negatively affecting methanotrophs in coculture. Ruiz-Ruiz, Gómez-Borraz, Revah, and Morales (2020) found that in cocultures with excess O_2, the green microalga *Scenedesmus obtusiusculus* (GM) slowed CH_4 degradation rates beween 27-43 % for an alkaliphilic methanotrophic bacterium consortium (AMB). In cocultures, CH_4 degradation was dependant on the

ratio of S. *obtusiusculus* and the methanotroph consortium. A 3:1 AMB:GM ratio showed the fastest CH_4 degradation rate and greatest methane removal (393 ± 0.013 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}, 65.3 % removal) when compared to a 1:1 (271 ± 0.054 mg_{CH4} $g^{-1}_{biomass}$ $d^{-}1$, 19.0% removal) and a 1:3 ratio (271 ± 0.054 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}, 13.0 % removal). The authors contend that innouculum ratios with excessive GM resulted in the microalga alkalizing the growth medium beyond the optimum pH for the bacteria consortium; thus slowing methane oxidation rates.

Photoautotrophs can also be negatively affected by methanotroph presence in cocultures, as coculturing with fast growing bacteria can outcompete microalgae leading to detrimental shading effects and resource scarcity (Padmaperuma, Kapoore, Gilmour, & Vaidyanathan, 2018). The microalga *Chlorella sorokiniana* has been found to have much slower apparent growth rate in cocultures with *Methylococcus capsulatus* (Rasouli *et al.*, 2018). During mixotrophic growth, *Galdieria sulphuraria* UTEX 2919 displayed substantially increased coproporphyrin excretion, slower growth rates and lesser biomass titre when cocultured with a bacterial community enriched from industrial wastewater. These detrimental effects were generally attributed to the additional O_2 consumption of the bacterial community (Zhu *et al.*, 2022).

The nature of the relationship between partners in coculture is influenced by environmental factors (from mutualistic to parasitic for example) and can commonly change (Goers, Freemont, & Polizzi, 2014; Hoek *et al.*, 2016; Hom, Aiyar, Schaeme, Mittag, & Sasso, 2015). In microalgae-bacteria interactions, reciprocal nutritional exchanges (e.g. cross-feeding) tend to be the basis of mutualistic relationships. Most commonly, microalgae provide O_2 and organic metabolites produced via oxygenic photosynthesis, while bacteria produce CO_2 and inorganic substances via respiration that sustain microalgal growth (Zhang *et al.*, 2020). However, these mutualisms can devolve into various degrees of antagonisms depending on factors such as light intensity, mixing, and temperature. Changes to these parameters can modify the interactions and/or community structure (Chen, Zhang, Song, Sommerfeld, & Hu, 2009; Hom *et al.*, 2015). In photoautotroph-methanotroph cocultures, possible changes in partner interactions with environmental factors or relative partner concentrations have not been widely studied. In most reported cases, when O_2 is limiting, methanotroph biomass production and/or CH_4 oxidation is enhanced by microalgae growth. This is the case for cocultures of the microalgae *Scenedesmus* sp. with the methanotroph *Methylocystis parvus* (van der Ha *et al.*, 2012) and the microalgae *Chlorella sorokiniana* with the methanotroph *Methylococcus capsulatus* (Rasouli *et al.*, 2018). For chemostat cocultures of the cyanobacterium *Synechococcus* with the methanotroph *Methylomicrobium alcaliphilum* 20z, Hill *et al.* (2017) reported than *M. alcaliphilum* biomass production rates were influenced by the input of light (and hence photosynthetic O_2 production by *Synechococcus*). However, in batch cocultures of the same pair, Badr *et al.* (2020) reported that the photoautotroph:methanotroph ratio did not influence growth, as the limiting factor was CH_4 transfer rather than O_2 availability. An interesting O_2 interaction was described by Li *et al.* (2022), who found that culturing the mixotrophic microalga *Scenedesmus obliquus* with *Methylocystis bryophila* diminished chlorophyll concentration but

increased microalgae cell density. There was also decreased expression of the Calvin Cycle and photosynthetic genes in the microalgae. It was found that the organic carbon released by *M*. *bryophila* triggered a switch from autotrophic to mixotrophic growth in *S. obliquus* which explained the increase in biomass. Thus, the pair adapted to O_2 availability. At low O_2 concentrations the microalgae tended to grow autotrophically and the methanotroph would convert CH_4 into CO_2, while at high O_2 concentrations, heterotrophic algal growth would dominate, the methanotroph would not grow, and release organic matter instead of CO_2 (Li *et al.*, 2022).

An obstacle to further understanding photoautotroph-methanotroph interactions is that most of the reported cocultures have been conducted in batch or semi-batch conditions (Badr *et al.*, 2022; Li *et al.*, 2022; Rasouli *et al.*, 2018; van der Ha *et al.*, 2011). Batch cultivations complicate the study of coculture interactions, since conditions change dynamically with time. Thus, it can be challenging to establish the underlying causality of a specific outcome. In this sense, to better understand the effects different growth conditions have on photoautotroph-methanotroph dynamics, continuous steady state cocultures (alongside parallel axenic controls) would be advantageous, as cultivation conditions can be easily controlled and maintained over prolonged periods.

Another problem with studying coculture interactions is the lack of rapid methods to quantify the relative abundance of each microorganism. Recently Badr *et al.* (2020) reported a computational (E-C) method to quantify the proportional biomass of individual members in methanotroph-photoautotroph cocultures that was based on the overall mass balance and individual growth stoichiometry coefficients. The E-C method requires measurement of total optical density, headspace gas composition (CH_4, CO_2 and O_2), and dissolved CO_2 concentrations. It was verified using two model cocultures with good results: *Methylomicrobium alcaliphilum* 20ZR with *Synechococcus* sp. PC7002 and *Methylococcus capsulatus* with *Chlorella sorokiniana*. Unfortunately, a major drawback of the E-C method is its reliance on gas and dissolved carbon dioxide (CO_2) concentrations. For small volume batch experiments, continuously monitoring the gas phase is impractical; as headspace samplings are taken, gases could be removed in such a way as to (without replacement) impact experimental conditions. Additionally, the authors recognized that changes to headspace pressure or gas flow rates could cause significant errors. As total gas production and consumption are not necessarily balanced, this would often be the case for several cocultures. Using the sampling method described by Stone, He, and Wang (2019) was offered as a solution, however this method involves injecting an inert gas (e.g. Ar) to account for pressure losses in batch, or as a tracer gas for continuous systems. Thus, this modified E-C method: 1) entails modifying the headspace gas composition (which is not desirable), 2) possibly interferes with GC measurements (as Ar, N_2, and He are often used as carrier gases); and 3) does not solve the problem if the cocultures produce more gas than they consume. The E-C method also assumes stoichiometric coefficients are readily available and constant regardless of axenic or coculture growth, which might not be the case. For example, in the cocultures of *M*. *bryophila* and *S. obliquus* described by Li *et al.* (2022),

the yield of biomass on CH_4 changed from 0.18 to 0.30 mol_C/mol_{CH4} as O_2 headspace concentration increased from 10 to 50 % v/v.

1.5 Single Cell Protein

World population increases and the growing demand for nutritional food requires an expansion in global protein production (Kerckhof *et al.*, 2021). Aquaculture provides ~ 50 % of the world's fish supply (Matassa, Boon, Pikaar, & Verstraete, 2016), and is the largest and fastest growing animal protein industry in the world (Jones, Karpol, Friedman, Maru, & Tracy, 2020) Feed, specifically proteins, dominate aquaculture costs, as plant based proteins are ill suited for aquaculture species, which are mostly carnivorous and require their diet to be 35 to 60 % crude protein. In contrast terrestrial livestock require around 12-26 % protein in their diets (Jones *et al.*, 2020). Soybean meal and fishmeal are common proteins used as animal feed (Kerckhof *et al.*, 2021). Fishmeal, which is produced from ground forage fish, fish trimmings and waste, is the preferred aquaculture feed due to its high protein content, high essential amino acid concentration, and quality of protein. Aquaculture accounts for 73 % of fishmeal consumption (Matassa *et al.*, 2016) and its production results in depletion of wild fish stocks (Kerckhof *et al.*, 2021). Other animal protein industries, like swine and poultry, compete for fishmeal use (Jones *et al.*, 2020). Alternative protein sources with lesser environmental impacts, both as human food and animal feed, are required to meet global protein demand.

The nutritional value of a protein feed is largely determined by its amino acid profile, specially the content of those amino acids that cannot be synthesised by humans or animals. These 'essential amino acids' include: phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine (Ritala, Hakkinen, Toivari, & Wiebe, 2017). The Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO) and the United Nations University (UNU) developed a standard that states the required daily intake of specific amino acids by humans (WHO/FAO/UNU Expert Consultation, 2007). This standard is often used in literature to evaluate if a protein source, consumed as the only dietary protein source, is sufficient to provide all the required indispensable amino acids for an adult (at a recommended protein intake level of 0.66 $g_{protein}$ kg^{-1} day^{-1}). THE FAO/WHO/UNU definition of indispensable amino acid includes the 'nutritionally indispensable' (e.g. the essential amino acids) and the 'conditionally indispensable' amino acids. These 'conditionally indispensable' amino acids are so named because they are recognised to possibly become indispensable under specific physiological or pathological conditions (e.g. cysteine, tyrosine, taurine, glycine, arginine, glutamine, and proline) (WHO/FAO/UNU Expert Consultation, 2007). Plant based proteins require less land, water, and are associated with lesser greenhouse gas emisions than animal based proteins, but their protein concentration and amino acid profiles don't compare favourably. Gorissen *et al.* (2018) compared several sources of plant based (e.g. oat, lupin, wheat, hemp, soy, brown rice, pea, corn, and potato) and animal based (e.g. whey, milk, caseinate, casein, and egg) protein isolates. While there was a wide variability in protein quantities and qualities between sources

and supliers, in general plant based protein isolates contained between 51 and 81 % protein, while animal based proteins ranged between 51 and 86 %. For plant based isolates, as an average, 26 ± 2 % of the total protein content were essential amino acids, which falls short of the WHO/FAO/UNU requirements (27.7%). Most of the plant based isolates were deficcient in isoleucine, valine, lysine, and methionine. Only the potato based protein satisfied all of the amino acid requirements and hence would be suitable as an exclusive protein source for human consumption. Animal-based protein isolates had an average amino acid content of 37 ± 2% of the total protein, and all analyzed sources met the WHO/FAO/UNU amino acid requirements, with the exception of the phenylanaline concentration in whey protein (3.5 % in the isolate vs 3.8 % requirement) (Gorissen *et al.*, 2018). Lysine is the most limiting essential amino acid in cereals, which are often fed to many animals, including pigs and poultry, but it is present in higher concentration in animal protein sources such as beef and egg (Erdman, Bergen, & Reddy, 1977; Skrede *et al.*, 1998).

Microorganisms typically posess faster rates of protein synthesis, and greater protein content than is observed in plants or animals (Table 1.1). They are also less nutritively demanding to grow, as they can often assimilate inorganic sources of nitrogen and/or carbon (Babel, Pöhland, & Soyez, 2000). Microbial biomass can be used as a nutritional source for humans and animals. Protein from microbial sources (e.g. bacteria, microalgae, yeasts, and fungi) is generally refered as Single Cell Protein (SCP), and has advantages over traditional sources of nutrition due to rapid growth/production rates, the abilility to grow on low-value or negative value waste substrates (e.g. CO_2 and CH_4), and/or reduced land-use and water-use requirements (Becker, 2007; Ritala *et al.*, 2017). SCP is often manufactured using abundantly available agricultural and industrial wastes (Anupama & Ravindra, 2000), and can be produced with a lesser environmental impact than, for example, soybean meal as a feed ingredient (Kerckhof *et al.*, 2021). Global demand for protein continues to drive interest in SCP, especially for those products with protein concentrations > 30 % and a favourable amino acid profile (Ritala *et al.*, 2017). Many SCP products, however, are often deficient in methionine (Erdman *et al.*, 1977; Skrede *et al.*, 1998).

1.5.1 Microalgal SCP

Microalgae are typically rich in protein, fats and vitamins (A, B, and C), contain 40–60% protein, 7% mineral salts, chlorophyll, bile pigments, fibre, and have low nucleic acid content (4–6% w/w) (Anupama & Ravindra, 2000). When used for animal or human consumption they typically have high protein content (60-70 %), low nucleic acid content (< 3 % w/w) and are sources of omega-3 fatty acids (Ritala *et al.*, 2017). Microalgae are mainly used as a source of omega fatty acids and carotenoids in aquaculture (Ritala *et al.*, 2017), as a liquid suspension to feed young fishes, and in dehydrated form to enrich feed for ornamental fish, crustaceans, shell fish, and bivalves (Oliveira, Monteiro, Robbs, & Leite, 1999). In general, most of the algal biomass that has been examined in literature (Table 1.2) has protein quality as good or better that conventional plant proteins, and compares favourably with other reference proteins (Becker, 2007). The main limitation to microalgal SCP for human consumption is the cellulose component of the algal cell wall, which cannot be degraded by humans (Anupama & Ravindra,

2000). This is also a problem in some feed applications, for example *Nannochloropsis ocultata* was found to be a poor fishmeal replacement for shrimp (Jones *et al.*, 2020). The cell wall however, does not pose an issue for cattle or other ruminants, as they host cellulose-degrading symbiotic bacteria and protozoa within their rumen (Anupama & Ravindra, 2000). Physical and mechanical methods can be used to rupture the cell wall, which improve digestibility and conserve the same nutrient and protein quality, but this greatly increases production costs (Jones *et al.*, 2020). Microbial contamination is also a problem as algal SCP production is often performed outdoors in open 'raceway' systems (Anupama & Ravindra, 2000).

Among exclusively photosynthetic microorganisms, the microalgae *Chlorella* sp. and *Scenedesmus obliquus*, as well as the cyanobacteria *Spirulina* sp. and *Athrospira* sp. have been heavily researched for large scale SCP production due to their high protein content (40-70 %) (Becker, 2007). Their essential amino acid composition is close to the requirements for animal feed, with minor deficiencies in methionine and cysteine (Roy & Pal, 2015). Typically methionine contents are ~ 1.4-2.6 %, and lysine 4.6-7.0 % (Anupama & Ravindra, 2000). *Spirulina* is the most extensively used microalgal SCP (Anupama & Ravindra, 2000). It is also one of the few SCPs commercialized as a human food supplement due to it's high protein content, 50 to 60 % (Templeton & Laurens, 2015). However it too possesses low concentrations of methionine, lysine and cysteine (Nyyssölä, Suhonen, Ritala, & Oksman-Caldentey, 2022).

For microalgae, cultivation parameters such as temperature, salinity, illumination, pH, mineral content, CO_2 supply, population density, growth phase and physiological status, can significantly influence macromolecular composition (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013). Despite this, the amino acid profile of algal SCP tends to vary minimally. For example, for *Chlorella* sp., at three different lifecycle stages in the course of nitrate replete to nitrate deprivation, only glutamic acid (a decrease from 12.4 to 10.5 % of the total aminoacid content), alanine (an increase from 8.6 to 10.8% of the total aminoacid content), and minor changes in lysine and arginine were detected, while the remaining amino acid content remained constant throughout all growth phases (Templeton & Laurens, 2015). Mixotrophic growth conditions affect lipid, carbohydrate and protein production differently in algae. For *Dunaliella salina*, protein production was enhanced up to 14-fold by glucose supplementation, while for *C. sorokiniana* the same glucose concentration only increased lipid content (Kadkhodaei *et al.*, 2015).

Red marine microalgae lack rigid cell walls, so their proteins are more easily digestable compared to microalgae with a cellulosic cell wall (Gaignard *et al.*, 2019). This makes members of this division as potentially attractive sources for SCP. Amoung the Rhodophyta, *Galdieria* spp. display an unusual macromolecular composition: high concentrations of carbohydrates and proteins, and very low lipid levels (Graziani *et al.*, 2013). Carbohyrates are important to the digestibility of algal biomass (Roy & Pal, 2015), so *Galdieria* spp. high carbohydrate content could be beneficial to its adoption as SCP. The

thermoacidophilic algae *Galdieria sulphuraria* has been suggested as a potential animal feed feedstock since it is rich in protein, phycocyanin, essential amino acids, and dietary fiber (Zhu *et al.*, 2022). It also has low contents of lipids and polyunsaturated fatty acids, which limit degradation and hence extend storage time (Graziani *et al.*, 2013). Its cultivation conditions (hot temperature and acidic pH) are expected to preclude microbial pathogen contamination.

A handful of studies have investigated *Galdieria* spp. for the production of SCP. Graziani *et al.* (2013) studied the differences between photoautotrophic and heterotrophic growth in *Galdieria sulphuraria*. Protein content was greater in photoautotrophically grown *Galdieria* compared to heterotrophic biomass (33 % vs 27 %). This difference was attributed to the loss of phycobiliproteins as a result of the absence of photosynthetic processes. Lipid concentrations for both culture conditions was very low, 1.1 % in autotrophic growth, and 1.4 % in heterotrophy. However, Barone *et al.* (2020) reported contradicting results: heterotrophically grown *Galdieria sulphuraria* contained 91 % more proteins, 57 % more lipids and 98 % more carbohydrates than photoautotrophically grown *Galdieria*. Barone *et al.* (2020) also reported that heterotrophic cells were larger, produced more stearic acid, oleic acid, monosaturated fatty acids, and ergosterol. Photoautotrophically cultured cells produced greater quantities of fatty acid methyl esters, saturated fatty acid and polyinsaturated fatty acids (Barone *et al.*, 2020). The difference in reported macromolecular contents could be atributed to differences in culture conditions. Microalgae are extremely sensitive to physical and chemical changes to their growth environment, which can lead to differences in growth and biochemical compositions (Salbitani & Carfagna, 2020). López *et al.* (2019) reported lipid contents of 15.34 ± 3.3 % for photoautotrophic, 3.64 ± 0.25 % for heterotrophic and 3.85 ± 0.13 % for mixotrophic *Galdieria* sp. USBA-GBX-832. Zhu *et al.* (2022) cultured mixotrophic *Galdieria sulphuraria* in wastewater media. Up to 68 % protein content was achieved in flask culture with a productivity of 2.12 g L^{-1} day^{-1}. In repeated fed batch cultures in a 5 L photobioreactor, biomass productivity was 12.0 g L^{-1} day^{-1}, with protein contents of 42.6-50.8 %, essential amino acids 18.9 %, lipids 21.9-39.1 %, and carbohydrates 5.96-6.02 %. The lesser protein content in the photobioreactor was attributed to a higher C/N ratio caused by glucose supplementation (Zhu *et al.*, 2022). From the amino acid profile of mixotrophically grown *G. sulphuraria* reported by Zhu *et al.* (2022), the microalgae meets the FAO/WHO/UNU standard for all indispensable amino acids with the exception of methionine and cysteine. Although it also compares negatively to other reported microalgae amino acid profiles, except for its greater content of tyrosine. Nevertheless, coculturing with wastewater bacteria was shown to improve the content of most indispensable amino acids, so it could be possible to further improve *Galdieria* spp. SCP quality by coculturing with other bacteria.

1.5.2 Methanotrophic SCP

Bacterial biomass has faster growth rates, typically has higher protein content, and more sulphur containing amino acids (e.g. methionine, cysteine and taurine) (Zha *et al.*, 2021) and lysine (Babel *et al.*, 2000) than other microbial (e.g. algal) biomasses. Bacterial SCP is typically abundant in protein (50-83%), with methionine contents ranging from 2.2-3.0 % and lysine 4.3-5.8 % (w/w) (Anupama &

Ravindra, 2000). Some comercially available bacterial SCP products include PRUTEEN, from Chemical Industries Ltd, that uses the methanol obligate bacteria *Methylophilus methylotrophus* (Overland, Tauson, Shearer, & Skrede, 2010) and Feedkind (Calysta) that uses the methanotroph *Methylococcus capsulatus* (Matassa *et al.*, 2016). The main disadvantage of bacterial SCP is the high concentration of nucleic acids in bacterial biomass (~15-16 % w/w) (Anupama & Ravindra, 2000). A diet rich in nucleic acids can lead to the acummulation of uric acid, which in turn may result in kidney stone formation and gout in humans, if its consumption is greater than the equivalent of 2 g day^{-1} of nucleic acids (Zepka, Jacob-Lopes, Goldbeck, Souza-Soares, & Queiroz, 2010). Bacterial SCP is also limited due to high costs associated with harvesting protein due to its small cell size (Anupama & Ravindra, 2000).

Of bacterial SCPs, methanotrophs have been extensively investigated because of their ability to convert low-value methane substrates into biomass. Methanotrophs, like other bacterial SCP, possess more easily digestable cell walls than most microalgae (Khoshnevisan, Tsapekos, Zhang, Valverde-Pérez, & Angelidaki, 2019) and can accumulate up to 70 % of their dry weight as protein (Tsapekos, Zhu, Pallis, & Angelidaki, 2020). Methanotrophs grown with natural gas have been suggested as sustainable protein sources for pigs, chickens, mink, fox, dogs, Atlantic salmon, rainbow trout, and Atlantic halibut; and have been found nutritionally favourable for feeding chickens and monogastric animals. Furtermore, The European Union has approved protein grown on natural gas to supplement feed for pigs, veal calves, and salmon (Overland *et al.*, 2010). Methanotroph growth rates are typically 0.085 h^{-1} for Verrucomicrobiota, 0.09 h^{-1} for Alphaproteobacteria, and 0.45 h^{-1} for Gammaproteobacteria (Tsapekos *et al.*, 2020). Yields vary with type of substrate, specific microorganism and the culture conditions maintained. Reported yield on methane vary from 0.15-1.78 g_{DW} g_{CH4}^{-1} depending on the microorganism and cultivation conditions (Khoshnevisan *et al.*, 2019). Overland *et al.* (2010) reported for *Methylococcus capsulatus* the production of 1 kg of crude protein from 1.7 kg of CH_4 using a specially designed aerobic loop fermenter. Commercial SCP from natural gas using *M. capsulatus* (Feedkind) has achieved productivities of 3-4 $kg_{protein}$ m^{-3} h^{-1} in continuous culture (Matassa *et al.*, 2016). Methanotroph production is hampered by gas transfer at commercial scale (Valverde-Pérez *et al.*, 2020), as a reference for *Methylocystis* sp., biomass concentrations of 25-65 g L^{-1} were achieved in continuous reactors (D = 0.17 h^{-1}) by using high CH_4 pressure to improve solubility (2.5 - 4 bar) (Wendlandt, Geyer, Mirschel, & Hemidi, 2005).

A bacterial protein meal produced by *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans*, *Bacillus brevis*, and *Bacillus firmus* grown on natural gas had an amino acid composition similar to fish meal, but with higher tryptophan (2.1 vs. 0.9 g/100 g protein) and lower lysine (6.1 vs. 8.6 g/100 g protein) contents. The amino acid profile was similar to other bacteria grown on methane (Skrede *et al.*, 1998). Overland *et al.* (2010) reported that SCP grown in natural gas had a similar aminoacid profile as the comercially available PRUTEEN, but with higher contents of arginine and tryptophan. Also, in terms of indispensable amino acids, differences with fishmeal and soybean meal were minimal, with the methanotoph containing less lysine and more tryptophan than fishmeal; and more methionine and

tryptophan than soybean meal. Variations in the amino acid composition among batches of the methanotroph were similar to the variations displayed by fishmeal and soybean meal (Overland *et al.*, 2010). Carere *et al.* (2019) measured the amino acid profile for chemostat cultures of *Methylacidiphilum* sp. RTK17.1 under variable O_2 and NH_4^+ conditions and reported only minor changes to the indispensable amino acid profiles between all conditions. *Methylacidiphilum* satisfied all the FAO/WHO/UNU standards, contained greater methionine, cysteine and lysine contents than most other SCPs, and was comparable to commercial fishmeal. *Methylacidiphilum* sp. RTK17.1 could, therefore, potentially be used as a SCP for feed applications.

The commercialisation of natural gas based SCP is challenged by the current low cost for soybean meal. The use of natural gas as a growth substrate also increases the carbon footprint of methanotroph SCP production (Matassa *et al.*, 2016). The carbon footprint of Feedkind®, for example, could be reduced from 5.8 ton CO_{2eq} ton^{-1} SCP to 1.7 ton CO_{2eq} ton^{-1} SCP if renewable sources of energy and biogas were used in the place of natural gas. Fishmeal and soybean meal have values of 2.6 and 0.8 ton CO_{2eq} ton^{-1} protein respectively (Matassa *et al.*, 2016). Cost of substrate has the greatest effect on profitability, followed by energy consumption (Babel *et al.*, 2000). Hence, using CH_4 from industrial waste gases could potentially help decrease costs for methanotrophic SCP production while simultaneously improving its environmental sustainability.

1.5.3 Coculture SCP

SCP produced from microbial cocultures offers the potential to improve protein quality by balancing the distribution of essential amino acids and by supplementing biomass with vitamins or lipids (Nyyssölä *et al.*, 2022). For example *Methylococcus capsulatus* has been cocultured with *Ralstonia* sp., *Brevibacillus agri*, and *Aneurinibacillus* sp. to permit ethane and propane assimilation, and enable growth with natural gas as a fermentation substrate in a continuous system (Overland *et al.*, 2010). However negative interactions between coculture partners can also occur, such as the production of inhibitory compounds or hostile environments (e.g. acidification) (Nyyssölä *et al.*, 2022; Zhu *et al.*, 2022).

Kerckhof *et al.* (2021) found that in various methanotroph and hydrogen oxidizing bacteria, half of the pairings (n =10) exhibited greater protein abundance than their axenic counterparts. Two cocultures were also reported to have an improved essential amino acid content than the respective synthetic mixture of axenic cultures. For the coculture SCP, protein concentrations ranged from 2.14-29.4 mg L^{-1} while the essential amino acid concentrations ranged between 2.16-14.5 $g/100g_{product}$. A combination of *Methyloparacoccus murrelli* LMG 27482 with *Cupriavidus necator* LMG 1201 had the best combination of protein content and amino acid profile. Feeding a human adult with this SCP was estimated to require 27-67 % less mass than chicken, whole egg, or tofu proteins. The protein, however, had lesser concentrations of histidine, lysine, and threonine than tofu and fishmeal. Coculturing also affected the amino acid distribution: cultivating a hydrogen oxidizing bacterium with a methanotroph increased the valine concentration in all cases, the isoleucine in 9 out of 10 cocultures, and the leucine

in 7 of 10 cocultures. For hydrogen oxidizing bacteria volumetric productivity as well as yield increased 2.8-4.8-fold when changing from sequential batch to continuous culture, and when *Methylococcus capsulatus* and *Methylomonas albus* were grown continuously aspartic acid, glutamic acid, valine, leucine, tyrosine, phenylalanine, lysine, arginine, methionine, and cystine contents increased (Kerckhof *et al.*, 2021).

Cocultures of *C*. *sorokiniana* and *M*. *capsulatus* had lower protein concentrations (28 % of the dry weight) that their respective axenic cultures (45 % and 53 % respectively), but higher fat contents (34 % for coculture, 30 % of the algae, and 22 % for the methanotroph). The consortium also had the highest carbohydrate concentration. *M*. *capsulatus* axenic protein content was also lower than what reported in literature (67-81 %), and the difference was attributed to industrial wastewater being the cultivation media, as typical synthetic media are richer in ammonia. Both microorganisms exhibited similar amino acid compositions to other commercialised protein sources, soybean meal, and bacterial proteins, and were deemed suitable for chicken and fish consumption (Rasouli *et al.*, 2018).

1.6 Research aims

The objective of this thesis is to investigate the suitability of coculturing thermoacidiphilic methanotrophs with microalgae to produce SCP. Preliminary evidence has suggested that *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 can co-exist, but nothing is known about the coculture's productivity, stability, nutritional suitability, or its ability to consume industrial waste gas emissions (e.g. petroleum, geothermal power station). Hence this research intends to study a consortium of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in order to develop a novel biotechnological platform that allows to convert low value gas waste streams into supplementary biofeedstocks. It is expected that the consortium will grow simultaneously using the waste streams substrates and the metabolic by-products generated by the other species in the consortium. Thus, this thesis will address the following hypotheses:

H1. *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 can grow together and maintain a stable coculture during both batch and continuous cultivation using substrates representative of industrial gas emissions (e.g. petroleum industry, geothermal power stations). The resulting biomass is predicted to have similar or superior quality as a biofeedstock to other commercial single cell proteins products.

H2. Co-cultivation of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 will exhibit a beneficial mutualistic relationship as a consequence of reciprocal O_2/CO_2 cross-feeding.

H3. Coculture performance (e.g. growth rates, biomass yields, CH_4/O_2 consumption/productivity rates) is dependent on the relative concentration of both *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1.

Table 1.1. Macromolecular composition of common microorganisms researched for SCP production and reference proteins.

Source	Macromolecular Content (%DW)				Source
	Protein	Lipids	Carbohydrates	Ash	
Reference proteins					
Fishmeal	73.8	12.01	NR	13.1	(Skrede *et al.*, 1998)
Microalgae					
Arthrospira maxima	62.8-70.2	6.0-7.3	9.9-19.6	NR	(Oliveira *et al.*, 1999)
Arthrospira maxima	60–71	6-7	13-16	NR	(Becker, 2007)
Arthrospira platensis	59.4-71.6	6.3-7.2	10.5-19.9	NR	(Oliveira *et al.*, 1999)
Chlorella vulgaris	42.0-55.2	3.51-17.0	NR	NR	(Safafar *et al.*, 2016)
Chlorella vulgaris (green)	38	5.1	19.9	24.2	(Batista *et al.*, 2013)
Chlorella vulgaris (orange)	12.3	27.6	22.7	34.8	(Batista *et al.*, 2013)
Chlorella pyrenoidosa	46.3-65.2	2.5-17.6	NR	NR	(Safafar *et al.*, 2016)
Chlorella sorokiniana	45.0	29.9	22.5	NR	(Rasouli *et al.*, 2018)
Dunaliella sp.	25.7-43.4	5.9-18.0	24.2-40.2	15.9-23.6	(Hossain & Mahlia, 2019; Moh. Muhaemin & Richardus, 2010)
Scenedesmus obliquus	50-56	12-14	10-17	NR	(Becker, 2007)
Scenedesmus sp.	35.0-50.7	8.0-26.7	NR	3.1-12.0	(Apandi, Mohamed, Latiffi, Rozlan, & Al-Gheethi, 2017; Hossain & Mahlia, 2019)
Spirogyra sp.	6-20	11-21	33-64	NR	(Becker, 2007)
Spirulina maxima	44.9	3.6	16.6	30.9	(Batista *et al.*, 2013)
Spirulina platensis	46–63	4-9	8-14	NR	(Becker, 2007)
Spirulina platensis	20.4-43.2	3.8-12.7	8.5-66.6	NR	(Hossain & Mahlia, 2019; Markou, Chatzipavlidis, & Georgakakis, 2012)
Synechococcus sp.	63	11	15	NR	(Becker, 2007)
Porphyridium cruentum	28-39	9-14	40-57	NR	(Becker, 2007)
Galdieria sulphuraria (Autotrophic)	32.5	1.8	62.9	NR	(Graziani *et al.*, 2013)
Galdieria sulphuraria (mixotrophic)	42.6-47.6	33.0-39.1	5.3-6.0	NR	(Zhu *et al.*, 2022)
Galdieria sulphuraria (Heterotrophic)	26.5	1.14	69.1	NR	(Graziani *et al.*, 2013)
Bacteria					
Bacterial Meal (methanotroph)	73.2	10.74	NR	8.5	(Skrede *et al.*, 1998)
Halomonas desiderata	15.4-44.9	6.7-42.1	NR	8.3-8.9	(Steinberg, Kronyak, & House, 2017)
Methylococcus capsulatus	40-51.5	25.5-36	NR	3.2-8.4	(Steinberg *et al.*, 2017)
Methylococcus capsulatus	52.52	21.82	16.36	NR	(Rasouli *et al.*, 2018)
Methylophilus methylotrophus	81.3	7.2	NR	9.1	(Overland *et al.*, 2010)
Thermus aquaticus	61	15.8	NR	20.7	(Steinberg *et al.*, 2017)
Cocultures					
Chlorella sorokiniana + *Methylococcus capsulatus*	27.62	34.5	31.96	NR	(Rasouli *et al.*, 2018)
Methylococcus capsulatus+ *Ralstonia* sp.+ *Brevibacillus agri* + *Aneurinibacillus* sp.	67.0-73.4	8.1-10.7	NR	6.2-8.5	(Overland *et al.*, 2010)
Galdieria sulphuraria + Wastewater bacteria (mixotrophic culture)	43.7-50.8	21.9-34.2	4.8-6.0	NR	(Zhu *et al.*, 2022)

NR stand for non-reported.

Table 1.2. Essential and indispensable amino acid content of reference proteins and common microorganisms researched for SCP production.

Protein Source	Amino Acid Content (g/100 $g_{protein}$)															Reference
	His	Ile	Leu	Lys	Met+ Cys	Met	Cys	Phe+ Tyr	Phe	Tyr	Thr	Trp	Val	Total EAA	Total IAA	
Reference Proteins																
Soybean Meal	2.7	4.7	7.5	6.1	2.8	1.3	1.5	8.8	5.0	3.8	3.9	1.4	4.8	37.4	42.7	(Overland *et al.*, 2010)
Fishmeal	2.5	4.7	7.9	8.2	3.9	3.0	0.9	7.3	4.1	3.2	4.0	0.9	5.3	40.6	44.7	(Overland *et al.*, 2010)
Whey	1.9	6.7	5.8	NR	NR	1.1	NR	NR	2.4	1.5	9.2	NR	6.8	NR	NR	(Schultz, Chang, Hauck, Reuss, & Syldatk, 2006)
Egg	2.4	6.6	8.8	5.3	5.5	3.2	2.3	10.0	5.8	4.2	5.0	1.7	7.2	46.0	52.5	(Becker, 2007)
Bean	2.6	5.3	7.7	6.4	3.2	1.3	1.9	8.7	5.0	3.7	4.0	1.4	5.3	39.0	44.6	(Becker, 2007)
FAO/WHO/UNU Standard	1.5	3.0	5.9	4.5	2.2	1.6	0.6	3.8	-	-	2.3	0.6	3.9	-	27.7	(Organization & University, 2007)
Microalgae																
Aphanizomenon sp.	0.9	2.9	5.2	3.5	0.9	0.7	0.2	2.5	2.5	NR	3.3	0.7	3.2	22.9	19.7	(Becker, 2007)
Arthrospira maxima	1.8	6.0	8.0	4.6	1.8	1.4	0.4	8.8	4.9	3.9	4.6	1.4	6.5	39.2	32.9	(Becker, 2007)
Chlorella vulgaris	2.0	3.8	8.8	8.4	3.6	2.2	1.4	8.4	5.0	3.4	4.8	2.1	5.5	42.6	35.4	(Becker, 2007)
Chlorella Vulgaris	2.0	4.0	9.5	6.1	4.0	2.4	1.6	10.2	6.0	4.2	4.8	2.1	6.0	43.0	48.8	(Templeton & Laurens, 2015)
Dunaliella bardawil	1.8	4.2	11.0	7.0	3.5	2.3	1.2	9.5	5.8	3.7	5.4	0.7	5.8	44.0	35.9	(Becker, 2007)
Nannochloropsis salina	1.8	5.2	9.8	2.9	3.3	2.3	1.0	11.0	6.8	4.1	5.9	1.7	7.1	43.7	48.8	(Templeton & Laurens, 2015)
Nannofrustulum	1.7	5.1	8.3	6.0	4.4	2.3	2.1	10.2	5.9	4.3	5.4	1.7	6.0	42.5	48.9	(Templeton & Laurens, 2015)
Neochloris oleoabundans	1.5	4.5	8.6	5.9	5.3	2.7	2.6	8.9	5.2	3.8	5.9	1.8	6.6	42.5	48.9	(Templeton & Laurens, 2015)
Phaeodactylum tricornutum	1.8	4.8	7.7	6.3	3.7	2.3	1.4	9.3	5.7	3.6	4.8	1.6	5.5	40.4	45.4	(Templeton & Laurens, 2015)
Scenedesmus obliquus	2.1	3.6	7.3	6.6	2.1	1.5	0.6	8.0	4.8	3.2	5.1	0.3	6.0	37.3	31.0	(Becker, 2007)
Scenedesmus sp.	1.2	4.7	9.7	4.9	5.7	3.1	2.6	10.0	6.3	3.6	6.5	2.2	6.7	45.3	51.5	(Templeton & Laurens, 2015)
Spirulina platensis	2.2	6.7	9.8	4.8	3.4	2.5	0.9	10.6	5.3	5.3	6.2	0.3	7.1	44.9	37.1	(Becker, 2007)
Galdieria sulphuraria (Mixotrophic)	1.7	4.0	6.7	6.4	1.4	1.3	0.1	8.5	4.0	7.1	4.7	0.9	5.3	35.1	39.7	(Zhu *et al.*, 2022)
Bacteria																
Undefined Bacterial Meal[a]	2.2	4.4	7.5	5.6	3.3	2.6	0.7	7.8	4.2	3.6	4.3	2.2	5.8	38.8	43.1	(Overland *et al.*, 2010)
Undefined Bacterial Meal[b]	1.9	4.3	7.0	6.0	3.1	2.4	0.7	7.5	4.1	3.4	4.6	0.9	5.6	36.8	40.9	(Overland *et al.*, 2010)
Methylacidiphilum sp. RTK17.1[c]	2.4	5.9	9.2	7.9	4.2	3.2	1.0	11.3	6.1	5.2	4.9	NR	6.5	46.0	52.2	(Carere *et al.*, 2019)
Methylacidiphilum sp. RTK17.1[d]	2.3	5.8	9.4	7.8	4.0	3.1	0.9	11.0	6.0	4.9	4.9	NR	6.2	45.4	51.3	(Carere *et al.*, 2019)
Methylacidiphilum sp. RTK17.1[e]	2.3	5.7	9.3	8.0	4.0	3.1	0.9	10.9	5.9	5.0	4.8	NR	6.0	45.1	51.0	(Carere *et al.*, 2019)
Methylacidiphilum sp. RTK17.1[f]	2.4	5.8	9.5	8.3	3.8	2.7	1.1	10.5	5.6	4.8	4.6	NR	5.9	44.8	50.7	(Carere *et al.*, 2019)

Table 2.2. (continued). Essential and indispensable amino acid content of reference proteins and researched for SCP production.

Protein Source	Amino Acid Content (g/100 $g_{protein}$)														Reference
	His	**Ile**	**Leu**	**Lys**	**Met+ Cys**	**Met**	**Cys**	**Phe+ Tyr**	**Phe**	**Tyr**	**Thr**	**Trp**	**Val**	**Total EAA**	**Total IAA**	
Coculture																
Galdieria sulphuraria + Wastewater bacteria	1.8	4.4	7.3	6.9	1.6	1.5	0.1	9.1	4.3	7.1	5.0	1.0	5.6	37.7	42.6	(Zhu *et al.*, 2022)
Methylocystis hirsute + *Paenibacillus* sp.	2.7	9.8	9.8	8.7	NR	NR	NR	NR	2.5	NR	5.3	NR	9.8	NR	46.0	(Kerckhof *et al.*, 2021)
Methylparacoccus murreli + *Cupriavidus necator*	3.1	8.2	16.1	9.2	NR	NR	NR	NR	3.6	NR	6.2	NR	12.2	NR	55.1	(Kerckhof *et al.*, 2021)

NR stand for non-reported. EAA stands for essential amino acids. IAA stands for Indispensable amino acids. [a]Cultured with natural gas. [b]Cultured with methanol [c]O_2 limited without addition of NH_4^+ (N_2 fixing) or H_2.[d]O_2 limited with addition of H_2 but not NH_4^+ (N_2 fixing).[e]O_2 limited with addition of NH_4^+ and H_2.[f]O_2 replete with addition of NH_4^+ and H_2.

In order to interrogate these hypotheses, four research objectives have been developed. This thesis will:

1. Test if *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 can be grown in stable batch and continuous coculture. Coculture growth and performance will be compared to axenic culture counterparts. The suitability of resulting SCP biomass will be evaluated against other single cell protein feedstocks.
2. Develop and validate a method to quickly measure the relative concentrations of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 using a combination of differential sedimentation, optical density, and fluorescence techniques.
3. Evaluate the influence of the *Methylacidiphilum*:*Galdieria* mass ratios on coculture performance, as well as the role of O_2 concentration in *Methylacidiphilum-Galdieria* interactions in batch cultures.
4. Study the interactions between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in low O_2 environments in steady state continuous coculture.

1.7 Thesis Outlook

In this section I briefly present the thesis structure and contents of this research.

Chapter 2 serves as a proof of principle for the simultaneous cocultivation of the thermoacidophilic methanotroph *Methylacidiphilum* sp. RTK17.1, and the microalga *Galdieria* sp. RTK37.1. In this chapter, I cultivate cocultures of *Methylacidiphilum* sp. RTK 17.1 and *Galdieria* sp. RTK37.1 in batch and continuous systems to assess their biomass productivity and performance. Coculture performance is compared to the corresponding axenic cultures and the nutritional suitability of resultant biomass as single cell protein feedstock is assessed. Stable coculture is achieved in both batch and chemostat systems. In batch, *Galdieria* enhances growth and methane oxidation rates of *Methylacidiphilum*, and complete methane removal is achieved without formation of an explosive mixture (as described by Janès *et al*., (2011)). In chemostat coculture experiments, *Galdieria* decreases net volumetric O_2 consumption but its oxygenic activity is unable to supply *Methylacidiphilum* with the O_2 required for complete CH_4 removal. *Methylacidiphilum* sp. RTK 17.1, *Galdieria* sp. RTK 37.1, and their coculture each display similar nutritional profiles, with protein quality comparable to soybean meal and fishmeal feeds used for animals. Nevertheless, *Methylacidiphilum* benefits from the presence of *Galdieria* in a low O_2 environment; with O_2 algae-methanotroph cross-feeding deemed to play a fundamental role on their interactions. With the exception of the nutritional analysis (Massey University), the *Methylacidiphilum* sp. RTK17.1 culture in 10 L STR reactor (Christophe Collet with assistance of Carlos Cartin Caballero), and the *Galdieria* sp. RTK37.1 culture in 80 L tubular photobioreactor (Daniel Smith with assistance of Carlos Cartin Caballero) all experimentation, data analysis, and write-up in this chapter was performed by Carlos Cartin Caballero.

In **Chapter 3** I develop a method for measuring the relative abundance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in cocultures, with a combination of differential sedimentation, optical density, and fluorescence (DSOF Method). The validity of the DSOF method is tested using artificial non-growing *Methylacidiphilum-Galdieria* mixtures (prepared from axenic stocks) across a wide range of defined biomass concentrations (OD_{600}). I determine that the absolute error of derived biomass concentration values using the DSOF method is ≤ ± 0.1 A.U. if [*Galdieria*] ≤ 2.0 A.U., and [*Methylacidiphilum*] ≤ 1.5 A.U.

The method is further evaluated on active cocultures under a variety of growth conditions. Derived *Methylacidiphilum-Galdieria* concentrations in these cocultures are consistent with their expected growth behaviour and prior observations. The DSOF method is determined to be a simple, rapid, and accurate method to quantify the relative concentrations of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture. All experimentation, data analysis, and write-up in this chapter was performed by Carlos Cartin Caballero.

In **Chapter 4** the influence of *Methylacidiphilum*:*Galdieria* mass ratios in coculture performance, as well as the role of O_2 concentration in *Methylacidiphilum-Galdieria* interactions in batch cultures is investigated. Several batch cocultures in serum bottles with variable initial *Methylacidiphilum*:*Galdieria* mass ratios (constant initial *Galdieria* sp. RTK37.1 and varying initial *Methylacidiphilum* sp. RTK17.1 concentrations) with and without CH_4 are performed. When using dilute initial concentrations of methanotroph, cocultures with CH_4 fix more net carbon. However, CH_4-containing cocultures with greater initial *Methylacidiphilum* concentrations collapse; with growth rates and net carbon fixation decreasing drastically. Coproporphyrin excretion is observed, and *Galdieria* suffers from chlorosis. By culturing additional batch cocultures in pressurized bottles under different conditions, I establish that chlorosis is caused by O_2 limitation, presumably due to *Methylacidiphilum*'s strong affinity for O_2. This chapter concludes by establishing that the O_2-limited chlorosis is reversible, as coproporphyrin is reabsorbed and pigments synthesised once *Methylacidiphilum* stops consuming O_2. With the exception on the LC-MS analysis (Harriet Newson) all experimentation, data analysis, and write-up in this chapter was performed by Carlos Cartin Caballero.

Finally, in **Chapter 5** the interactions between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in low O_2 environments during steady state continuous coculture are investigated. By changing the O_2 inlet concentration of steady state continuous axenic cultures and cocultures I establish that O_2 concentration and net uptake/production rates determine the nature of *Methylacidiphilum-Galdieria* interactions in cocultures. In chemostat coculture at dilute O_2 concentrations, the interaction becomes inhibitory for the microalga: *Methylacidiphilum* benefits from *Galdieria* O_2 production, while at the same time harming the microalgae by inducing O_2-limitation chlorosis. When O_2 inlet concentration is increased, the relationship becomes neutral for the microalga: *Methylacidiphilum* grows faster due to improved O_2 availability, allowing CH_4 to be oxidized further, without negatively impacting *Galdieria*. Considering these results, I continuously modified conditions in a fed-batch coculture in such a way as to obtain a high biomass concentration coculture. With the exception of the nutritional analysis (Massey University), all experimentation, data analysis, and write-up in this chapter was performed by Carlos Cartin Caballero.

In conclusion, **Chapter 6** presents a summary and conclusions of this thesis along with the recommendations for future work and study.

Chapter 2. Simultaneous co-cultivation of the thermoacidophilic methanotroph, *Methylacidiphilum* sp. RTK17.1, and the microalga, *Galdieria* sp. RTK37.1, in batch and chemostat reactors

Abstract

The verrucomicrobial methanotroph, *Methylacidiphilum* sp. RTK17.1, and the unicellular red alga, *Galdieria* sp. RTK37.1 are both thermoacidophilic microorganisms isolated from the Rotokawa geothermal area in Aotearoa-New Zealand**.** As thermoacidophiles they are likely to grow under industrially relevant conditions: hot temperatures due to the bubbling of hot flue gases, high CO_2, and acidic pH. In coculture microalgae provide O_2 and organic substances through photosynthesis, and bacteria produce CO_2 and inorganic substances through respiration that sustain microalgal growth (Zhang *et al.*, 2020). Thus a consortium of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 could potentially be used to convert CH_4 and CO_2 in waste gas streams into valuable products, like single cell protein for animal feed. In this work, cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 were cultivated in batch and continuous systems to assess their biomass productivity and performance. Coculture performance was compared to corresponding axenic cultures and the nutritional suitability of resultant biomass as single cell protein feedstock was assessed. Stable coculture was achieved in both batch and chemostat systems. In serum bottle batch experiments, *Galdieria* enhanced growth (29 %) and methane oxidation (300 %) rates of *Methylacidiphilum* significantly (*p*-values < 0.05 and < 0.001 respectively), and complete methane removal was achieved without formation of an explosive mixture. I found no evidence of microalgae being negatively affected by the methanotroph presence. In steady state chemostat coculture experiments, *Galdieria* decreased net volumetric O_2 consumption by 46 %, but its oxygenic activity was unable to supply *Methylacidiphilum* with the O_2 required for complete CH_4 removal. Nevertheless, *Methylacidiphilum* benefits from the presence of *Galdieria* in a low O_2 environment; with O_2 algae-methanotroph cross-feeding deemed to play a fundamental role on their interactions. *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1, and their coculture each displayed similar nutritional profiles, with protein quality comparable to soybean meal and fishmeal feeds used for animals. The biomass needed to meet the daily indispensable amino acid requirements of a 62 kg human was found to be: 568 g for *Methylacidiphilum*, 804.8 g *Galdieria*, and 753.7 g for the coculture, with histidine being the limiting amino acid. These thermoacidophilic cocultures, which have not previously been investigated, offer great potential to convert low (or negative) value industrial gas streams into valuable products (e.g. supplementary biofeedstocks).

2.1 Introduction

Methane (CH_4) is the most abundant organic gas in the atmosphere (Hanson & Hanson, 1996), and is the main contributor to climate change gas after carbon dioxide (CO_2) (Cantera, Munoz, *et al.*, 2018). While CO_2 abatement has been the main focus of climate change control strategies, CH_4 emission reduction has been proposed as an important additional strategy (Hamer, 2010). Aerobic methane oxidizing bacteria (methanotrophs) can consume CH_4 as their sole carbon and energy source (Hanson & Hanson, 1996), and they serve as the primary CH_4 biological sink (Chistoserdova, 2015; Hanson & Hanson, 1996). Methanotrophs could potentially use the CH_4 in waste gas streams for growth, and the produced biomass

could be used as single cell protein (SCP) for animal feed (Strong *et al.*, 2016; Strong *et al.*, 2015). Biomass from the methanotroph *Methylococcus capsulatus* biomass is comercially available as SCP (Matassa *et al.*, 2016), and using natural gas as microbial feedstock has been successful in producing feed for the farmed salmon market (Hamer, 2010). Methane oxidation by aerobic methanotrophs requires O_2 supplementation, which presents safety concerns for bioprocess development as CH_4-air mixtures become explosive between 5% and 15% CH_4 by volume (Fei *et al.*, 2014). A potential solution to this problem is to coculture methanotrophs with oxygenic photoautotrophic microalgae; as oxygen could be produced and consumed *in situ* simultaneously, thus minimising the possible development of an explosive gas (CH_4-Air) mixture.

Interactions between species in cocultures can be stimulating, inhibitory, or neutral (Singh, Ryu, & Kim, 2019). Stimulating interactions are usually established nutrition exchange (Zhang *et al.*, 2020), where one member produces materials that the other member uses, and they include mutualism (where each member benefits from the association) and commensalism (where one member benefits while the other one is not affected). Inhibitory interactions usually arise from resource and/or spatial limitations, and can result in production of secondary metabolites that give an advantage to one of the species in detriment of the other (Zhang, Merino, Okamoto, & Gedalanga, 2018). Methanotrophic bacteria have previously been shown to benefit from simultaneous cultivation with other microorganisms. For example, Jeong *et al.* (2014) found that *Sphingopyxis* sp. NM1, a heterotrophic alphaproteobacterium, stimulates the growth of the methanotroph *Methylocystis* sp. M6 in a concentration dependant manner. A volumetric ratio of 1:9 (methanotroph:heterotroph) was found to increase methane oxidation 1.34 times, and *Methylocystis* population density 2.4 times (Jeong, Cho, & Kim, 2014). A similar effect was found in cocultures of *Hyphomicrobium* sp. with *Methylocystis* sp. (Jeong & Kim, 2018). However, while the oxidation rate and biomass of the methanotroph increased, *Hyphomicrobium* populations tended to decrease with time (Jeong & Kim, 2018). The cell masses of both the bacteria *Rhizobium* sp. Rb122 and the methanotroph *Methylovulum miyakonse* HT12 increased when co-cultured in batch; cobalamin production by *Rhizobium was* found to be the growth stimulating factor (Iguchi, Yurimoto, & Sakai, 2011). In other instances, methanotrophs benefit from coculture by removal of harmful or inhibitory substances by their partners, for *Methylococcus capsulatus* to use natural gas as a substrate in continuous system over long periods of time, it requires coculture with *Ralstonia* sp., *Brevibacillus agri* and *Aneurinibacillus* sp. to remove accumulated acetate and other inhibitory organic material (Bothe *et al.*, 2002).

In photoautotroph-bacteria interactions nutrition exchange tends to be the base of mutualistic relationships, most commonly microalgae provide O_2 and organic substances through photosynthesis, and bacteria produce CO_2 and inorganic substances through respiration that sustain microalgal growth (Zhang *et al.*, 2020). There are several examples in literature of coculture of non-extremophilic methanotrophs with photoautotrophs, for example, van der Ha *et al.* (2011) cocultured unspecified methane oxidizing communities with microalgae communities obtained from open ponds. It was found that this methanotroph-photoautotroph coculture had a faster CO_2 fixation rate and required 40-55 % less oxygen supplementation, but methane oxidation rates were not significantly affected by the microalgae. As another example, *Methylococcus capsulatus* and the microalgae *Chlorella sorokiniana* were used to recover nutrients by producing single cell proteins from potato processing wastewater (Rasouli *et al.*, 2018). The coculture had

a slower maximum observed growth rate, lesser protein content, and greater fat and carbohydrate contents compared to axenic cultures (Rasouli *et al.*, 2018). Hill *et al.* (2017) cultured *Methylomicrobium alcaliphilum* 20z with the cyanobacteria *Synechococcus* PCC 7701 using biogas with no added O_2. It was found that the batch coculture had higher biomass loads but slightly lower specific growth rates than the axenic *Methylomicrobium*, while the axenic *Synechococcus* showed the fastest growth rate and greatest biomass concentrations of the 3 cultures. Furthermore, in illuminated chemostat coculture using synthetic natural gas as a growth substrate, the cyanobacterium produced enough O_2 to support growth with negligible dissolved O_2 concentrations (Hill *et al.*, 2017). A significant obstacle to implementing any of the aforementioned cocultures at industrial scale is that typical flue gas concentrations of CO_2, NO_x and SO_x can quickly acidify the coculture growth media, resulting in inhibited growth and diminishing productivity (Jiang *et al.*, 2013).

Most microalgae grow well at CO_2 concentrations ranging between 1-5 % (v/v) in the gas phase (Ota *et al.*, 2015) but at concentrations > 10%, CO_2 may be toxic to some species (Cheng *et al.*, 2016). Industrial flue gases contain CO_2 at typical levels of 10-12 % and may contain SO_2 in concentrations that, even with efficient scrubbing technologies, reach 2400 ppm (Varshney, Mikulic, Vonshak, Beardall, & Wangikar, 2015). SO_2 hydrolyzes in water, and the released H^+ acidifies the media (Zhao & Su, 2014). At an aeration rate of 0.25 vvm, flue gases containing 100-250 ppm of SO_2 have been found to acidify media to 2.5 - 3.5 (Lee, Lee, Shin, Park, & Kim, 2000). This creates a key challenge for industrial bioprocesses seeking to scale-up microalgae under industrially relevant conditions: hot temperatures due to the bubbling of hot flue gases, high CO_2 concentration, and acidic pH. While few algae are viable at these conditions, the thermoacidophilic red algae *Galdieria* spp. are often found in similar hostile environments like volcanic hot sulphur springs and solfatara soils (Marquardt & Rhiel, 1997; Vítová *et al.*, 2016).

Extremophilic methanotrophs and microalgae have been found in geothermal environments (Donachie, Christenson, Kunkel, Malahoff, & Alam, 2002; Op den Camp, Mohammadi, Pol, & Dunfield, 2018). Thus, a coculture of extremophilic methanotrophs and algae could potentially be used to convert waste gas streams into single cell protein for animal feed. In this study, the coculturing of thermoacidophilic methanotroph and algae strains was investigated. The verrucomicrobial methanotroph, *Methylacidiphilum* sp. RTK17.1, and the microalga, *Galdieria* sp. RTK37.1 were both isolated from soils within the Rotokawa geothermal area in Aotearoa-New Zealand**.** *Methylacidiphilum* sp. RTK17.1 grows optimally at pH 2.5 and 50 °C temperature (T_{max} = 60 °C), oxidizes CH_4, reduces CO_2 via the Calvin-Benson-Bassham cycle, fixes N_2 under nitrogen limiting conditions, accumulates glycogen, and rapidly consumes H_2 under microaerophilic conditions (Carere *et al.*, 2017). The microalgae *Galdieria* sp. RTK37.1 has not been widely characterized; however, genomic sequencing indicates it is closely related to *Galdieria sulphuraria* (Buckeridge, 2022). *G. sulphuraria* has a temperature tolerance of 56 °C, a maximum tolerance of 100% CO_2 concentration (Varshney *et al.*, 2015), and grows in pH values between 0 and 4 (Vítová *et al.*, 2016). The verrucomicrobial methanotroph *Methylacidiphilum* sp. RTK17.1 could potentially grow in co-culture with the acidophilic microalga *Galdieria* sp. RTK37.1, however no prior studies on the coculturing of thermoacidophilic methanotrophs and microalgae exist. Furthermore, little is known about the differences between their axenic and co-culture growth, their possible mutualistic relationships, or their suitability for feed

applications. Therefore, the aim of this study is to assess if *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 could be grown in coculture, to compare the differences between their axenic and coculture growth, and to test their suitability as single cell protein feedstocks.

2.2 Materials and Methods

2.2.1 Growth Media and Stock Culture Maintenance

A modified V4 media (Dunfield *et al.*, 2007) was used for all cultures. Briefly, the media contained per litre: 0.4 g NH_4Cl, 0.05 g KH_2PO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 3 mL FeEDTA solution, 3 mL trace elements solution, 1 mL trace metals solution, 0.2 µM $Ce_2(SO_4)_3$, and 0.2 µM $La_2(SO_4)_3$ solution. The media was adjusted to pH 2.5 with H_2SO_4. Refer to Appendix A for details on media and solution preparation.

Methylacidiphilum sp. RTK17.1 was routinely maintained in chemostat culture using a 1 L bioreactor (BioFlo 110; New Brunswick Scientific, Edison, NJ, United States) with a 600 mL working volume and 0.0069 h^{-1} dilution rate. A gas mixture (69 % CO_2, 1.0 % CH_4, 3.1 % O_2, balance N_2, all v/v) was supplied at 20 mL min^{-1}. Temperature was maintained at 50 °C, agitation at 800 rpm, and pH ~ 2.5 (but not controlled). Under these conditions, typical OD_{600} values were 1.2 A.U. and outlet gas concentrations were 72 % CO_2, 0.13 % CH_4, 1.5 % O_2, with the remaining attributed to N_2. For all experiments, *Methylacidiphilum* sp. RTK17.1 was aseptically harvested directly from the reactor and diluted with fresh V4 media to the desired starting concentration (as determined spectrophotometrically at 600 nm).

For *Galdieria* sp. RTK37.1, 300 mL stocks were routinely grown in batch cycles within 1 L Duran Pressure Plus Bottles equipped with bromobutyl rubber stoppers. At the beginning of each cycle, the microalgae would be diluted back to OD_{600} 1.0 with fresh V4 media, the bottles were then subjected to a vacuum for 3 minutes, and re-pressurized to 5 psia with an 80% v/v CO_2 and 20% v/v N_2 gas mixture. The bottles were incubated horizontally in a Lab Companion shaking incubator (Cole-Parmer, Illinois, United States) at 110 rpm and 45 °C. Light was provided from the top by a lamp with three 50 W halogen lightbulbs and a dimmer. Light intensity was adjusted to 60 µmol photons m^{-2} s^{-1} measured at bottle's level with a LI-250A Light Meter (LI-COR, Nebraska, United States). Growth was stopped at OD_{600} 6.0 A.U. and the cycle started anew. For all experiments, *Galdieria* sp. RTK37.1 was aseptically harvested at a OD_{600} 5.0 A.U. and diluted with fresh V4 media to the desired starting concentration.

2.2.2 Coculture Batch experiments

Methylacidiphilum sp. RTK17.1 and *Galdieria* sp. RTK37.1 were grown together in batch to assess coculture viability and to investigate biomass productivity differences between coculture and axenic controls. For these experiments, 250 mL of sterile V4 media was added to 1 L Duran Pressure Plus Bottles sealed with bromobutyl rubber stoppers. For the cocultures, *Galdieria* sp. RTK37.1 was inoculated to a starting OD_{600} of 0.9 A.U. and *Methylacidiphilum* sp. RTK17.1 to OD_{600} 0.1 A.U. (Refer to Appendix B for OD_{600} to dry weight conversion factors). The axenic microalgae controls were inoculated to a starting OD_{600} of 1.0 A.U. and the axenic methanotroph controls to OD_{600} 0.1 A.U. Coculture headspaces were prepared by subjecting the bottle to vacuum for 3 minutes and then re-pressurizing to 5 psia with an 80% CO_2 and

20% N_2 gas mixture (v/v). The bottles were subjected to vacuum and repressurized 3 times. After this, headspace gas was equilibrated to atmospheric pressure by expanding against a syringe, and then 120 mL of 100% CH_4 was injected. The headspace gas was then released to 10 kPa. The axenic *Galdieria* sp. RTK37.1 headspace was prepared in a similar fashion, but without CH_4 addition. The headspace air was not replaced for the *Methylacidiphilum* sp. RTK17.1 controls, but 120 mL of CH_4 and 60 mL of CO_2 were injected, and then the pressure released to 10 kPa. Table 2.1 shows the headspace composition for the cultures, some residual O_2 is left in the *Galdieria* sp. RTK37.1 cultures and in the cocultures. All cocultures and controls were run in duplicates.

Table 2.1. Initial CO_2, CH_4, and O_2 headspace gas concentrations for the batch coculture experiments.

Culture	Headspace concentrations[a] (% v/v)		
	O_2	**CH_4**	**CO_2**
1. Methylacidiphilum sp. RTK17.1 (axenic)	13.44 ± 0.02	11.96 ± 0.33	5.12 ± 0.18
2. Coculture	1.38 ± 0.19	12.37 ± 1.28	64.05 ± 4.05
3. Galdieria sp. RTK37.1 (axenic)	1.41 ± 0.01	0	77.90 ± 2.36

[a] Balance N_2 for all cultures.

All bottles were incubated horizontally in a shaking incubator at 110 rpm and 45 °C. Light was provided from the top of the shaker by a lamp with three 50 W halogen lightbulbs and a dimmer. Light intensity was adjusted to 60 µmol photons m^{-2} s^{-1} measured at bottle level. To assess cell growth and gas production/consumption rates respectively, 2 mL liquid and 10 mL gas samples were taken daily. Headspace pressure was measured with an Almelmo 5470 data logger (Ahlborn, Germany) equipped with a pressure sensor (Gems Sensors & Controls, England) before and after gas sampling to account for gas losses. To minimise glycogen accumulation under N-depletion (Carere *et al.*, 2019), experiments were stopped when ammonium was depleted, or the stationary phase was reached. To harvest the biomass for nutritional analysis, the replicates were combined and centrifuged at 12,000 rpm on a 5810R Benchtop Centrifuge (Eppendorf, Hamburg) for 15 minutes. Supernatants were discarded, and each biomass pellet was stored at -20 °C until needed. To assess the effect on glycogen accumulation on the microalgae's protein concentration, 3 additional bottles of *Galdieria* sp. RTK37.1 were grown, as described above, but allowed to reach stationary phase (OD_{600} = 9.25 ± 0.3 A.U.). Insufficient axenic *Methylacidiphilum* sp. RTK17.1 biomass was obtained from these batch experiments to allow for its nutritional characterization.

2.2.3 Chemostat Coculture Experiments

To evaluate if chemostat coculture of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 was possible, as well as to generate sufficient biomass for amino acid characterization, a continuous reactor was set up for each axenic culture and coculture. For each (3 total), a 1 L bioreactor (BioFlo 110, New Brunswick Scientific, Edison, NJ, USA) was used, cultures were incubated at 45 °C and V4 media at pH 2.5 was used. Bioreactor working volume was maintained at 900 mL by automatic regulation of the culture level with a scavenging/effluent pump. For the axenic methanotroph and the coculture, media was supplied, and spent broth withdrawn at a constant flow rate of 150 mL day^{-1} (D= 0.167 day^{-1}), and the agitation was kept constant at 250 rpm. Due to technical and time constraints, microalgal chemostat cultivation was performed at different agitation (400 rpm) and dilution rates (media fed and withdrawn at 250 ml day^{-1}, D =

0.278 day^{-1}). Dissolved O_2 was monitored using an InPro 6810 Polarographic Oxygen Sensor (Mettler-Toledo, Columbus, OH, USA). pH was monitored with a Polilyte Plus pH Meter (Hamilton Company, United States). Custom gas mixtures were prepared in compressed gas cylinders and supplied at 16 ml min^{-1} using mass flow controllers (El-flow, Bronkhorst, Netherlands). Feed gas was filter sterilised before entering the reactors using in-line 0.22 µm hydrophobic filters (MicroScience, Australia). Feed gas composition for *Methylacidiphilum* sp. RTK17.1 and the coculture were approximately 2.1 % O_2, 1.1 % CH_4, 74 % CO_2, balance N_2, (all v/v). For *Galdieria* sp. RTK37.1 feed gas composition was 3.0 % O_2, 64 % CO_2, balance N_2. Light was supplied to the axenic microalgae and coculture via warm white LED strips attached to the walls of the reactor. Light intensity was adjusted to 100 µmol photons m^{-2} s^{-1} measured at the centre of the empty reactor with a LI-250A Light Meter (LI-COR, Nebraska, United States). To initiate axenic culture experiments, reactors were inoculated to OD_{600} 0.1 A.U. with the corresponding microorganism and grown in batch mode until an OD_{600} of 1.0 A.U. was reached; after which the inlet and outlet peristaltic pumps were activated (Cole-Parmer, Illinois, United States). For the coculture experiment, the reactor was inoculated to 0.1 OD_{600} of *Galdieria* sp. RTK37.1, grown in batch until OD_{600} = 1.0 A.U. and then the pumps were started. After achieving steady state, < 1 mL *Methylacidiphilum* sp. RTK17.1 (OD_{600} = 1.20 A.U.) was added to the reactor. For all reactors 2 mL liquid and 40 mL inlet and outlet gas samples were harvested daily using aseptic technique for biomass (OD_{600}) and gas measurements. Systems were considered at steady state when there was less than a 5 % OD_{600} change over a five day period. For nutritional analysis the steady state outflow broth was collected daily and stored at 4 °C. After 1 L of broth had accumulated, it was centrifuged at 12,000 rpm for 15 minutes. Supernatants were then discarded, and the resulting biomass pellets were stored at -20 °C until needed.

2.2.4 *Methylacidiphilum* sp. RTK17.1 culture in 10 L STR reactor

In order to produce sufficient biomass (> 5 g_{DW}) to allow for complete amino acid characterization (including tryptophan) in batch culture, *Methylacidiphilum* sp. RTK37.1 was cultivated in a 10 L STR bioreactor. 1 L of the effluent from the *Methylacidiphilum* sp. RTK17.1 chemostat described in section 2.2.3 was used to inoculate a 10 L Biostat D-DCU STR reactor (Sartorius, Germany) with a 10 L working volume. Prior to inoculation, the reactor and V4 media were sterilized for 20 min at 121 °C. A custom gas mixture (12.3 % CO_2, 2.5 % CH_4, 7.9 % O_2, balance N_2, all v/v) was supplied continuously at 800 mL min^{-1}. The inlet gas mixture was sterilized via a Sartufluor GA Cartridge inline single-layer PTFE membrane filter (Sartorius, Germany). Throughout the cultivation (220 h total) temperature was maintained at 50 °C, agitation at 500 rpm, and pressure at 100 mbar. To ensure cells never experienced N-limitation, 4 g of NH_4Cl dissolved into 50 mL deionized water was added at OD_{600} = 3.0 A.U. (120 h) and OD_{600} = 6.0 A.U. (198 h). Following 220 h, 9.1 L were harvested (OD_{600} = 9.2 A.U.). This harvested broth was then centrifuged for 10 min at 8500 rpm on a Sorvall RC6 Plus centrifuge (Thermo Scientific, United States) and subsequently stored at -80°C for 24 h to prepare for freeze drying. Finally, the cell mass was freeze dried for 72 h using an Edwards SuperModulyo Freeze Dryer (Edwards, Stockholm), and then stored at -20 °C until needed for nutritional analysis.

2.2.5 *Galdieria* sp. RTK37.1 culture in 80 L tubular photobioreactor

Galdieria sp. RTK37.1 was cultivated in an 80 L tubular photobioreactor, as described by Smith (2020), to produce the biomass required (> 5 g_{DW}) for complete amino acid characterization (including tryptophan) in batch culture. Briefly, the microalgae were first cultivated in 250 mL baffled shake flasks (100 mL working volume, V4 media, 45 °C, pH 2.5) inside a MaxQ 6000 shaking incubator (Thermo Fisher Scientific, USA). The incubator was modified with LED lights (warm white, 103 $umol_{photons}$ m^{-2} s^{-1}) and a gas manifold system able to continuously supply air supplemented with CO_2 at 3% (25 mL min^{-1}). When *Galdieria* OD_{600} reached 6.4 A.U., it was used to inoculate 1350 mL of sterile V4 media in a 1.5 L concentric tube airlift reactor**.** An air and CO_2 mixture was supplied via mass flow controllers (MCS series, Alicat Scientific, USA) (sparging rate 0.3 V_{gas} $V^{-1}_{reactor}$ min^{-1}, final CO_2 concentration 3 %), and humidified by bubbling the gas mixture through deionised water in a Buchner flask. A 0.2 µm inline filter was used between the humidifying Buchner flask and the reactor**.** Warm white light was supplied from LED strips coiled around an acrylic tube located concentrically around the reactor, with a 119 $umol_{photons}$ m^{-2} s^{-1} intensity. When OD reached 6.6 A.U., the entire contents of the airlift reactor were used to inoculate an 80 L tubular photobioreactor.

The custom 80 L tubular photobioreactor consisted of a bubble column and a solar collector (Smith, 2020). The bubble column had an internal diameter of 140 mm and a height of 1800 mm and was equipped with a ring-type sparger at the centre of the base of the column, a stainless-steel heat exchanger, and pH, D.O., and temperature probes. The final working volume of the reactor was approximately 76 L with 23 L in the bubble column and 53 L in the solar receiver. The solar receiver was constructed of 12 borosilicate glass tubes (Schott, Germany) of 1500 mm length and 50.4 mm internal diameter. The culture was circulated using a variable-speed centrifugal pump. Lighting was provided by RGBWW LED strips attached to the inside of 100 mm PVC pipe which was split lengthwise to be able to clam shell around each of the solar receiver tubes. Each lighting tube contained a total length of 19.5 m of LED strips. Air and CO_2 flow rates were controlled with mass flow controllers (Alicat Scientific, USA). Air was supplied from an air compressor and food grade CO_2 was supplied from cylinders (BOC, New Zealand). The air and CO_2 mixture was filtered with an inline 0.2 µm PTFE filter (WhatmanTM Polycap 75 TF, GE Healthcare, USA) prior to entering the bubble column.

To prepare the V4 growth medium, water and concentrated chemical stock solutions were transferred via a peristaltic pump from 20 L plastic carboys, and sterilized via an inline 0.2 µm filter. The media was then circulated though the tubes at 0.6 m s^{-1} liquid velocity. Compressed air and CO_2 were added at a total flowrate of 7.6 L min^{-1}, and final CO_2 concentration of 3 %. The media was circulated for at least an hour with bubbling before inoculation, to ensure that it was well mixed, and at the correct temperature (45 °C). Light intensity was set to 100 $umol_{photons}$ m^{-2} s^{-1}, and then increased to 300 $umol_{photons}$ m^{-2} s^{-1} after 5 days of culture. On day 19, prior to ammonium depletion (OD_{600} = 3.6 A.U.), 24 L were harvested. In order to analyse macromolecular and amino acid contents of N-depleted *Galdieria* sp. RTK37.1, the reactor was topped up to 76 L with reverse osmosis water. The culture was allowed to continue for an additional 7 days, and then harvested in its entirety on day 26 (OD_{600} = 4.7 A.U.). Next, the harvested *Galdieria* (both N-excess and N-limited) were separately vacuum filtered, and the collected biomass paste washed with deionised water. The washed biomass paste was scraped from the filter, collected, and stored overnight at

–80 °C, before freeze drying using a Freezone 2.5 freeze-dryer (Labconco). Finally, the freeze-dried biomass was stored at -80 °C until needed for nutritional analysis.

2.2.6 Analytical procedures

Optical density at 600 nm was routinely measured using an Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom). To estimate biomass concentrations in the batch bottle experiments, conversion factors previously obtained in axenic cultures were used. One unit of OD_{600} was equivalent to 0.308 g_{DW} L^{-1} for *Galdieria* sp. RTK37.1 (Appendix B.1, Figure B.1) and to 0.435 g_{DW} L^{-1} for *Methylacidiphilum* sp. RTK17.1 (Appendix B.2, Figure B.2). For the coculture, relative amounts of methanotroph and microalgae were estimated using the oxygen uptake/production rate of the axenic cultures and the oxygen production of the coculture**.** For the chemostat reactors, biomass concentrations were measured gravimetrically. For this, after steady state was achieved, 50 mL aliquots were taken and centrifuged at 12,000 rpm for 15 minutes. Supernatants were discarded, and the pellets were resuspended in deionized water and centrifuged again for 15 minutes. Supernatants were discarded and the pellets were transferred to pre weighted aluminium dishes and left drying overnight at a 95 °C oven. The aliquot biomass weight was calculated by difference. Technical triplicates were measured.

Gas samples (Batch experiments: headspace; chemostat experiments: influent, effluent) were analysed for CO_2, CH_4, N_2, and O_2 concentrations using a 490 micro GC equipped with a thermal conductivity detector (Agilent Technologies, United States) with Molecular Sieve 5A with a heated injector (50 °C, back-flush at 5.10 s, column at 90 °C, 150 kPa), a PoraPak Q column with a heated injector (50 °C, no back-flush, column at 70 °C, 50 kPa) and a 5CB column with a heated injector (50 °C, no back-flush, column at 80 °C, 150 kPa).

Ammonium concentrations were monitored as described using the orthophthaldialdehyde (OPA) method (Taylor, Ninjoor, Dowd, & Tappel, 1974) on liquid samples filtered through 0.2 µm nylon syringe filters**.** Briefly, buffered phthalaldehyde-mercaptoethanol reagent was prepared by mixing 4.5 mL of phtaladehyde solution (10 mg mL^{-1} in 100% ethanol), 4.5 mL of mercaptoethanol solution (5 µL mL^{-1} in 100% ethanol), and 81 mL of 0.2 sodium phosphate buffer (pH = 7.4). A 50 µL aliquot of sample was added to 1.45 mL of the buffered OPA reagent. The mixture was allowed to stand 45 minutes at room temperature. The fluorescence was then measured at a 410 nm excitation and 470 nm emission in a spectrophotometer (Varioskan Lux, Thermo Scientific, United States). The ammonia fluorescence was corrected for the fluorescence of a deionized water blank. A calibration curve with ammonium chloride standards was run with every set of samples.

Nutritional characterization of biomass (ash, crude protein, fat, carbohydrates, and amino acid profile) was performed by the Massey University Nutrition Laboratory according to the official methods of analysis of the Association of Official Analytical Communities (AOAC, 2005) international. Ash content was determined by the furnace method (AOAC method 9442,05), total crude protein was determined via the Dumas method (AOAC method 968.06), fat content was determined by the Mojoinnier method (AOAC method 922.06), and carbohydrates were determined by difference. Amino acid profile determination of acid-stable residues

was performed via reverse-phase high performance liquid chromatography (HPLC) separation using AccQ derivatization of biomass (60–140 mg) samples following oxidization with performic acid, and hydrolysis with hydrochloric acid as described in AOAC method 994.12 (AOAC, 2005). Cysteine and methionine content was determined by performic acid oxidation (AOAC method 985.28).

All Statistical analysis were performed using Prism Graphpad 9.4.1. Indispensable Amino Acid contents were compared using unpaired two-tailed *t*-tests (α = 0.05). For all other analysis, unless stated otherwise, Two-factor ANOVA (alpha = 0.05) tests were used, with Sidak method for multiple mean comparisons between columns (simple effects within rows) or rows (simple effects within columns). Growth rates were calculated by fitting the appropriate concentration data to the exponential growth equation model in Prism Graphpad 9.4.1 ($Y = Y_0 e^{\mu_{max} t}$, where Y is the concentration in A.U., Y_0 is the concentration at time zero in A.U., μ_{max} is the specific growth rate in h^{-1}, and t is time in h).

2.3 Results and Discussion

2.3.1 Batch coculture experiments

To assess coculture viability and to study differences between coculture and axenic growth, *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 were cultivated within gastight bottles in batch mode. The objective of these early experiments was to ascertain whether the oxygenic activity of *Galdieria* sp. RTK37.1 could support the *Methylacidiphilum* sp. RTK17.1-dependent aerobic oxidation of CH_4, without the need for supplemental O_2 addition. Axenic methanotroph batch cultures reached stationary phase following 4 days incubation with an OD_{600} of 0.40 ± 0.03 A.U. (Figure 3.1). As expected, the onset of stationary phase corresponded to an O_2 limitation in the headspace (0.9 % O_2) (Figure 2.2a). In the coculture, *Galdieria* sp. RTK37.1 was inoculated to a starting OD_{600} of 0.9 A.U. (0.277 g_{DW} L^{-1}) and *Methylacidiphilum* sp. RTK17.1 to 0.1 A.U. (0.045 g_{DW} L^{-1}). The axenic microalgae controls were inoculated to a starting OD_{600} of 1.0 A.U. (0.308 g_{DW} L^{-1}) and the axenic methanotroph controls to 0.1 A.U. (0.045 g_{DW} L^{-1}). Optical density measurements indicate growth on the coculture with OD_{600} values comparable to the microalgae control, 4.05 ± 0.21 A.U. (1.34 ± 0.07 g_{DW} L^{-1}) and 3.95 ± 0.07 A.U. respectively (1.22 ± 0.02 g_{DW} L^{-1}) (Figure 2.1). This contrasts with the results from coculture of *Galdieria sulphuraria* UTEX 2919 with a bacterial community (predominately Proteobacteria, Actinobacteria, and Acidobacteria) from industrial wastewater, where biomass concentration decreased from 12.85 ± 0.20 g L^{-1} for axenic mixotrophic *Galdieria* to 11.47 ± 0.25 g L^{-1} in coculture, this was attributed to the bacterial community increasing shading and O_2 limitation (Zhu *et al.*, 2022).

This study represents the first report of thermoacidophilic methanotroph-photoautotroph coculturing. The specific growth rate for *Methylacidiphilum* sp. RTK17.1 in coculture (0.022 h^{-1}) was significantly faster than in the axenic control (0.017 h^{-1}) (*p*-value < 0.05). In other (non-thermoacidophilic) methanotroph-photoautotroph cocultures, the photoautotroph tends to have a beneficial effect on the methanotroph, e.g**.** faster growth and/or CH_4 oxidation rates; which is attributed to an increase in O_2 supplementation due to photoautotroph photosynthetic activity (Badr *et al.*, 2022; Badr *et al.*, 2020). van der Ha *et al.* (2011) reported a 1.6 time increase in biomass yield of a methane oxidizing community from anaerobic sludge

tanks when cocultured with a microalgal community dominated by *Scenedesmus* sp. The coculture of alkaliphilic *Methylomicrobium alcaliphilum* 20z with the cyanobacterium *Synechococcus* PCC 7002 had higher biomass concentrations and slightly slower specific growth rates (OD_{730} = 0.532 ± 0.017A.U., μ = 0.061 ± 0.005 h^{-1}) than the *M*. *alcaliphilum* controls, but the axenic *Synechococcus* cultures had the highest specific growth rate and biomass concentrations (OD_{730} = 0.545 ± 0.006 A.U., μ = 0.050 ± 0.002 h^{-1}) (Hill *et al.*, 2017). The biomass of a batch coculture including *Methylococcus capsulatus* and the microalga *Chlorella sorokiniana* was 2.17 times more concentrated in the stationary phase than their axenic counterparts (1488.2 mg L^{-1} vs 683.25 for C. *sorokiniana, and 263.56* mg L^{-1} for *M*. *capsulatus).* However, the apparent growth rates in cocultures were much slower compared to axenic controls (0.21 day^{-1} for the coculture vs 3.67 day^{-1} for C. *sorokiniana, and* 4.35 day^{-1} for *M*. *capsulatus).* Additionally, the coculture removed less chemical oxygen demand and phosphorus from the wastewater than the axenic cultures, but more ammonium, than *C. sorokiniana* (Rasouli *et al.*, 2018).

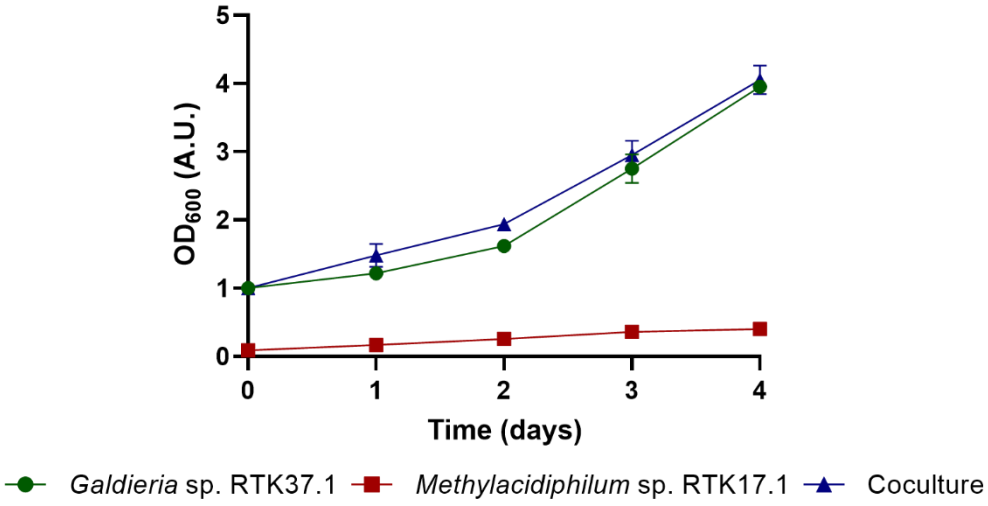


Figure 2.1. Batch coculture growth of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 (Coculture) compared to corresponding axenic control experiment. All cultures were grown in 1 L gastight bottles on a shaking incubator at 45 °C, pH 2.5, 110 rpm, and 60 μmol m^{-2} s^{-1} measured at the top of the bottle wall. The average value of duplicate experiments (n = 2) is shown with error bars representing one standard deviation.

The gas concentration profiles for the axenic cultures and the cocultures indicate O_2-dependent cross feeding enhances CH_4 removal in batch experiments (Figure 2.2). In the axenic cultures, O_2 is steadily accumulated by the microalga and depleted by the methanotroph (Figure 2.2a); while in the coculture the O_2 concentration remains stable (between 1.4-3.4 % v/v) until CH_4 is depleted on day 3 (Figure 2.2b). The cessation of CH_4 oxidation in the coculture by *Methylacidiphilum* coincides with rapidly increasing O_2 concentrations. The methanotroph quickly becomes limited by O_2 during axenic growth, and when active in cocultures, impedes O_2 gas accumulation in coculture with *Galdieria*. This behaviour is further evidenced by the CO_2 concentration profiles (Figure 2.2c). As CO_2 is required for photoautotrophic growth and produced via CH_4 oxidation, one would expect a steady CO_2 increase for the axenic methanotroph, and a stable concentration until day 3 followed by a quick decrease on the coculture. Although high CO_2 concentrations have been found to inhibit microbial growth in cocultures of *M. alcaliphilum* 20z with *Synechococcus* PCC 7002 (Hill *et al.*, 2017), *Methylacidiphilum* spp. and *Galdieria* spp. are reported to

grow without inhibition on up to 92.7 % and 100 % CO_2 environments respectively (Khadem, van Teeseling, *et al.*, 2012; Varshney *et al.*, 2015). Neither the axenic *Galdieria*, nor the coculture showed signs of CO_2 inhibition.

Methane is the energy source for *Methylacidiphilum* sp. RTK17.1, so its consumption is necessary for the methanotroph to remain anabolically active. In the axenic *Methylacidiphilum* sp. RTK17.1 batch culture experiments, CH_4 was slowly consumed until day 4, when O_2 reached 0.9 % v/v, with 49.5 % of methane removed. No further growth or change in gas concentrations was observed in the subsequent days (data not shown); therefore, a minimum headspace O_2 concentration appears necessary to sustain methane oxidation. In contrast, in the coculture experiments CH_4 oxidation was enhanced by the continuous O_2 production from *Galdieria* sp. RTK37.1. Consequently, CH_4 was completely removed from the headspace (LDL < 0.01 % CH_4) within 3 days incubation. As < 2 % residual O_2 was initially present in the coculture, the stoichiometric O_2 requirement for CH_4 oxidation dictates that both *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 were metabolically active in the coculture. Therefore, not only is coculture possible, but the methanotroph seems to benefit from this interaction, at least in low O_2 environments. The coupling of *Galdieria*'s oxygenic photosynthesis and *Methylacidiphilum's* methane oxidation in batch experiments also allowed for complete methane removal without formation of an explosive mixture (as defined by Janès *et al.*, 2011). This type of positive interaction has been reported for other methanotroph/photoautotroph cocultures. Hill *et al.* (2017) found that for a coculture of *Methylomicrobium alcaliphilum* 20z and *Synechococcus* PCC 7002 using raw biogas, longer periods of growth were possible than when compared to axenic controls, however specific growth and biomass concentration were lower for the coculture when compared to the axenic *Synechococcus.* Also, methane oxidation was possible without supplemental O_2 addition in both batch and chemostat cultivation. On the other hand, other methanotroph/photoautotroph cocultures have reported diminished productivity. For instance, Rasouli *et al.* (2018) reported methane consumption for *M. capsulatus* was slowed down when cocultured with *Chlorella sorokiniana,* even though oxygen was being continuously accumulated during the experiment. Lower O_2 concentrations at the beginning of culture as well as copper competition with the microalga were given as possible explanations for the slowed methanotroph dynamics.

In this study ammonium (NH_4^+) was used as the primary anabolic nitrogen source for both microorganisms. *Methylacidiphilum* spp. have been shown to fix N_2 under conditions of reduced-N limitation when O_2 concentrations are < 2 % v/v (Khadem, van Teeseling, *et al.*, 2012). Furthermore, both *Galdieria* spp. and *Methylacidiphilum* spp. are known to accumulate glycogen and have lesser protein yields under nitrogen starvation (Khadem, van Teeseling, *et al.*, 2012; Martinez-Garcia *et al.*, 2016; Sinetova *et al.*, 2006). As either N_2-fixation and/or glycogen production would complicate comparisons between cultures, experiments were stopped once ammonium was depleted. Ammonium depletion happened within 4 days of incubation for the coculture and axenic *Galdieria* experiments (Figure 2.2d), so it is possible some glycogen accumulation took place. However, it is unlikely that any N_2-fixation occurred as O_2 concentrations were > 2 % (v/v). At first glance it might be striking that there was no difference in the ammonium profiles between coculture and axenic microalgae experiments, but this make sense as there is little difference between the starting biomass concentrations of both cultures. The small change in ammonium concentration for the

axenic methanotroph is explained by the low initial biomass concentration of methanotroph; ~ 7-fold less than the microalgae or coculture.

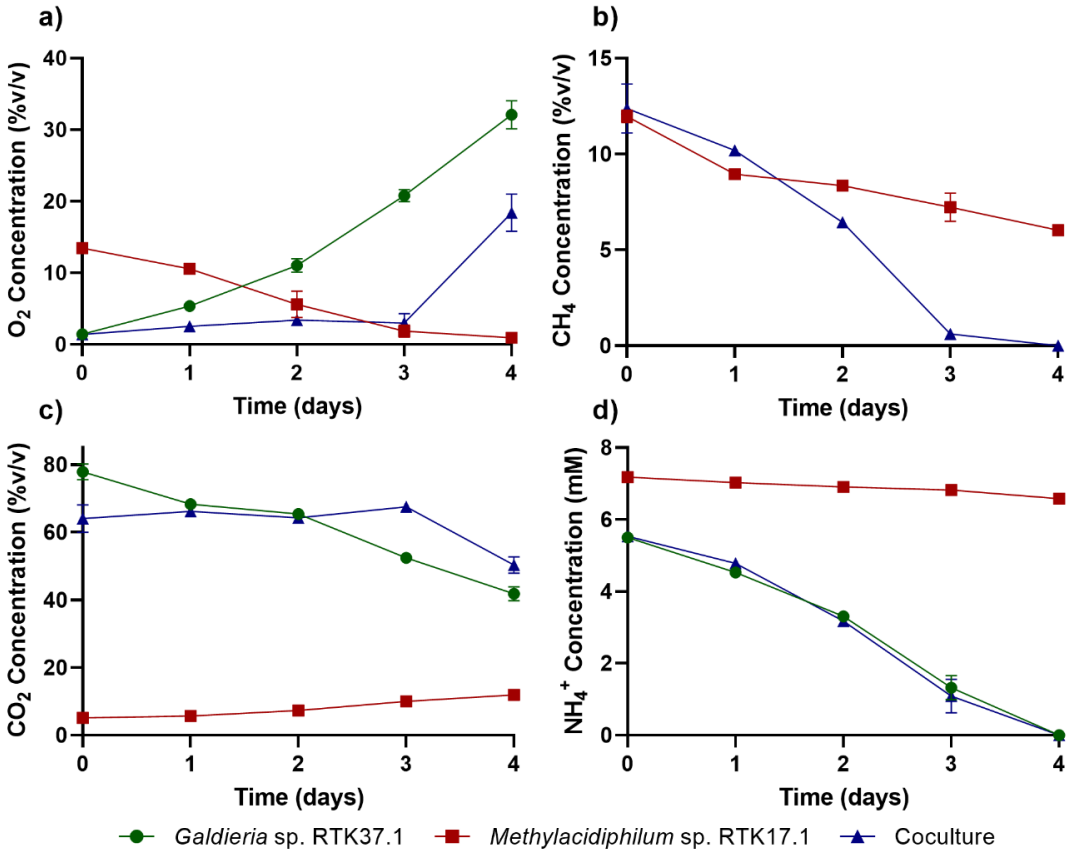


Figure 2.2. Concentration profile comparison between cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 (Coculture) and their axenic controls. a) O_2 headspace concentration. b) CH_4 headspace concentration. c) CO_2 headspace concentration. d) NH_4^+ concentration. All cultures were grown in 1 L gastight bottles on a shaking incubator at 45 °C, pH 2.5, 110 rpm, and 60 µmol $m^{-2}s^{-1}$ measured at the top of the bottle wall. The average value of duplicate experiments (n=2) is shown with error bars representing one standard deviation.

To better understand the differences in behaviour between the coculture and the axenic cultures, production and consumption rates were derived (Figure 2.3). The methanotroph and coculture share the same trend, an increasing O_2 consumption rate (negative O_2 production) with time, until day 3 when CH_4 and O_2 limitations affect the coculture and methanotroph respectively. There is a small net O_2 production (Figure 2.3a) at the start of coculture, which decreases to a net consumption by day 3, as CH_4 oxidation consumes most of the O_2 produced by the microalga. This is interesting considering than more O_2 was produced by the axenic microalga than what was consumed by the axenic methanotroph, which suggests that *Galdieria's* presence promotes *Methylacidiphilum*'s growth. However, this also means that O_2 could rapidly become a limiting factor in a coculture, if increased methane removal rates or more concentrated broths would be desired. Since a 0.9% O_2 headspace concentration completely inhibited growth and gas consumption/production in the axenic methanotroph, it is reasonable to assume the same would happen with the methanotroph in the coculture once CH_4 was depleted. When this happened, shortly after day 3, the O_2 production rates for coculture and axenic microalgae equated (*p*-value > 0.05), which suggests that the presence of *Methylacidiphilum* did not inhibit *Galdieria*'s photosynthetic capabilities, as they remain the

same with or without the bacterium. While O_2 production/consumption was balanced in the coculture as long as there was methanotrophic activity, a modest net CO_2 (Figure 2.3c) fixation was observed. This is consistent with reports that verrucomicrobial methanotrophs fix CO_2 via the Calvin Benson cycle (Hou *et al.*, 2008; Khadem, van Teeseling, *et al.*, 2012). Thus, a proportion of the CO_2 produced during CH_4 oxidation in coculture is assimilated into both new *Methylacidiphilum* sp. RTK17.1 and *Galdieria sp.* RTK37.1 biomass.

Methane consumption rates within axenic *Methylacidiphilum* sp. RTK17.1 cultures (Figure 2.3b) slowed following the first day, presumably due to O_2 limitation in the liquid phase, and ceased after day 4. In contrast, in cocultures CH_4 oxidation rates increased continuously until CH_4 was depleted shortly after day 3 (the apparent decrease between days 3 and 4 is a consequence of CH_4 depletion). This enhanced rate occurred even though at similar headspace O_2 concentrations methane oxidation stopped in the axenic *Methylacidiphilum*. I theorize that oxygenic photosynthesis in the coculture increases O_2 availability in the liquid phase, thus allowing for faster rates of CH_4 oxidation. With the exception of the first and last day of culture (when CH_4 oxidation stopped due to O_2 or CH_4 depletion), CH_4 consumption rates were 4-fold faster in the coculture than in the *Methylacidiphilum* axenic control (p-value < 0.001). Ammonium consumption rates (Figure 2.3d) were steady for *Methylacidiphilum* sp. RTK17.1 cultures and relatively slow when compared to *Galdieria* and coculture experiments, thus the presence of the methanotroph does not significantly impact the coculture's ammonium uptake (p-value > 0.05).

Figure 2.4 shows the specific consumption and production rates (O_2, CO_2, CH_4, NH_4^+) for the axenic cultures and the coculture. *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 biomass concentrations in the coculture were estimated by performing an oxygen balance each day, under the assumption that the specific O_2 uptake rates in the coculture were the same as in the axenic cultures (Figure 2.4a, an average of the specific O_2 consumption/production rates for days 1 to 3 was used). As CH_4 was completely consumed in the coculture shortly after day 3, I attributed any increase in OD_{600} for that period solely to *Galdieria* sp. RTK37.1. The observed specific O_2 production rates (Figure 2.4a) were relatively stable for the coculture and axenic controls up until the onset of nutrient limitation (O_2 for the methanotroph, NH_4^+ for the microalgae, and CH_4 for the coculture), which could be attributed to physiological changes in response to said limitations. This approach to approximate individual biomass concentrations in the coculture would then not work past day 3 of coculture, or under nutrient limitation. Oxygen specific uptake for the methanotroph was ~4-fold greater than microalgae specific production, which is further evidence that O_2 production could become the limiting factor to obtain higher density cocultures. When CH_4 was depleted, the coculture's O_2 specific production approximated that of the axenic *Galdieria* control; presumably due to *Methylacidiphilum* sp. RTK17.1's diminished activity in the absence of CH_4. It is worth noting that this was also the case for CO_2 (Figure 2.4c) and NH_4^+ (Figure 2.4d) specific consumption rates. Collectively, these data further suggest that *Galdieria* sp. RTK37.1 is not negatively affected by the presence of *Methylacidiphilum* sp. RTK17.1 in coculture.

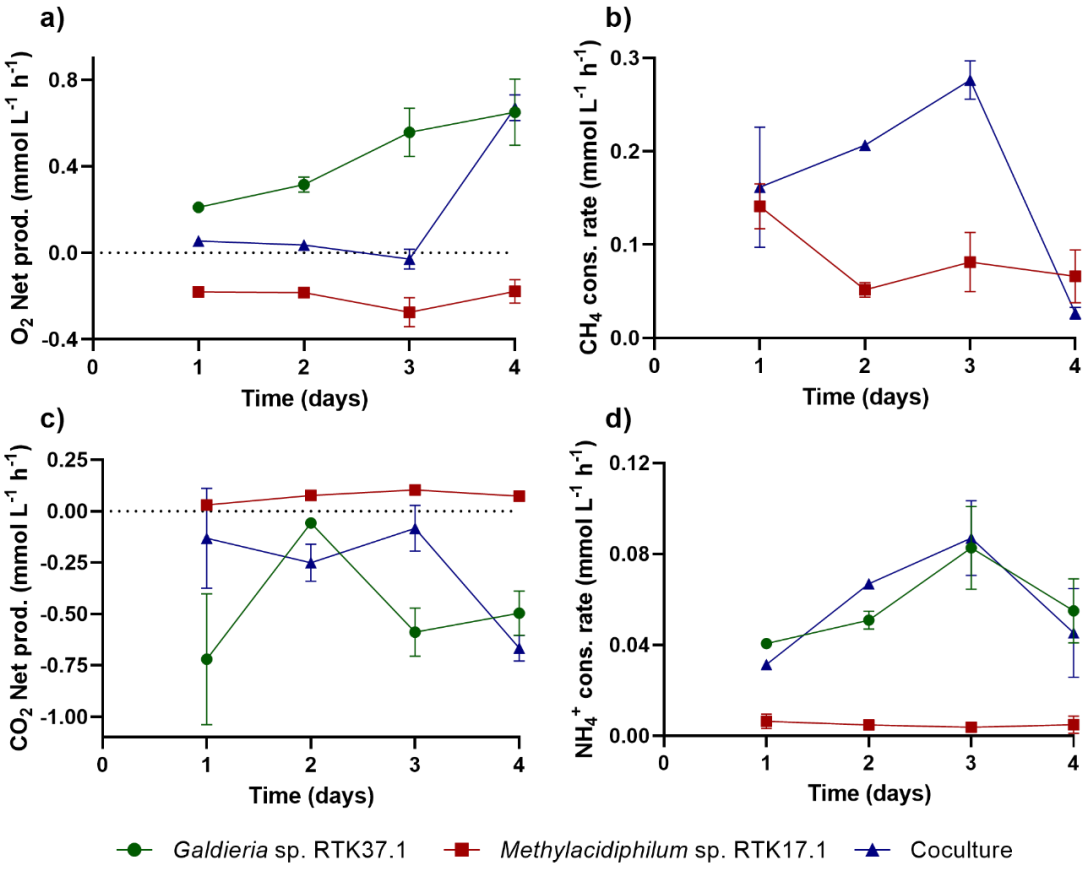


Figure 2.3. Production and consumption rates comparison between cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 (Coculture) and their axenic controls. a) O_2 net production rates. b) CH_4 consumption rates. c) CO_2 net production rates. d) NH_4^+ consumption rates. Cultures grown on 1 L gastight bottles on a shaking incubator at 45 °C, pH 2.5, 110 rpm, and 60 µmol m^{-2} s^{-1} warm white light (measured at the top of the bottle wall). The average value of duplicate experiments (n=2) is shown with error bars representing one standard deviation.

The CH_4 specific consumption rates (Figure 2.4b) suggest that enhanced CH_4 consumption in the coculture was caused both by an increase in *Methylacidiphilum's* biomass and by an increase in the amount of CH_4 consumed per mass unit of methanotroph (the dashed blue line in Figure 2.4b). Presumably the greater O_2 availability in cocultures facilitates faster CH_4 consumption rates. When calculated based on the total coculture biomass, the specific rate of CH_4 consumption for the coculture is slower than for the axenic methanotroph. This makes sense as the majority of coculture biomass consists of (non-methanotrophic) *Galdieria* sp. RTK37.1. However, it is curious that this specific rate was constant. A possible explanation is that as there was almost no O_2 in the headspace, methane fixation was limited by O_2 production, which is directly linked to microalgae growth. As *Galdieria* grew, more O_2 was available for *Methylacidiphilum*, which quickly utilised it to oxidise CH_4 for growth. Thus, CH_4 oxidation in coculture incubation was reliant on microalgae growth. Such a phenomena has been observed by Hill *et al.* (2017) with *M. alcaliphilum* biomass production rate being controlled by *Synechococcus* in an O_2 limited chemostat coculture. To prove this hypothesis however, a more accurate method to rapidly quantify the relative abundance of each microorganism would be required, as specific rates values could be muddled by innacurate biomass concentrations.

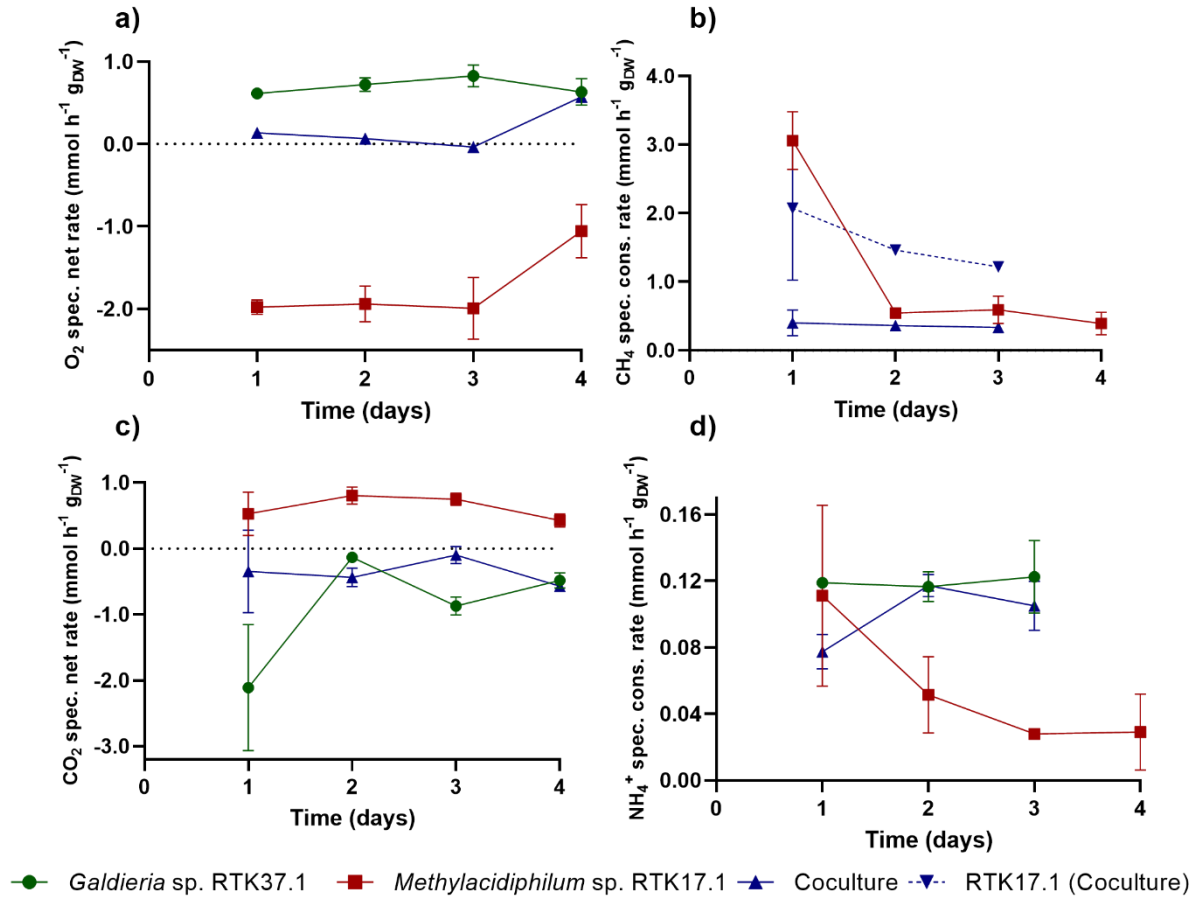


Figure 2.4. Specific production rates comparison between cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 (Coculture) and their axenic controls. Negative values indicate consumption rates, while positive values indicate production rates. Coculture concentrations were estimated by considering each microorganism individual specific rate to be the same as in their axenic culture a) O_2 specific production rates. b) CH_4 specific production rates. For clarity day 4 values for the coculture were omitted as their value drops due to methane depletion. c) CO_2 specific production rates. d) NH_4^+ specific production rates. For clarity, day 4 values for the methanotroph and the microalgae were omitted since their value drops to 0 due to ammonium depletion. Cultures grown in 1 L gastight bottles sideways on a shaking incubator at 45 °C, 110 rpm, and 60 µmol m^{-2} s^{-1} measured at the top of the bottle wall. The average value of duplicate experiments (n=2) is shown with error bars representing one standard deviation.

2.3.2 Chemostat coculture experiments

To evaluate if chemostat coculture of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 was possible (in addition to generating sufficient biomass for amino acid profile characterization), a continuous bioreactor was set up for each axenic culture and coculture. Due to technical and time constraints, different agitation and dilution rates for the microalgae reactor were used. Steady state was achieved in all three bioreactors (Table 2.2). To the best of my knowledge, this is the first reported instance of chemostat cultivation of a verrucomicrobial methanotroph with a photoautotroph in coculture.

The presence of *Galdieria* sp. RTK37.1 in the coculture increased the amount of dissolved oxygen in the broth, and decreased volumetric O_2 net consumption by 46%, when compared to the axenic *Methylacidiphilum*. However, unlike the batch coculture, in chemostat the microalga was unable to support the oxygen demand for the methanotroph; as the specific net O_2 production was -0.54 mmol h^{-1} g_{DW}^{-1}. This

observation is likely a consequence of the different *Galdieria:Methylacidiphilum* proportions in chemostat. In the batch coculture experiments, the *Galdieria:Methylacidiphilum* ratio was approximately 3.6, while in the chemostat it was ~1.3 (if we asume identical methanotroph biomass concentrations in axenic and coculture bioreactors). Coculture chemostat operation seems to require at least some external oxygen supplementation. This result contrasts with Hill *et al.* (2017) chemostat culture of *M*. *alcaliphilum* and *Synechococcus*, where the photosynthetic O_2 production was matched by O_2 consumption, so no O_2 supplementation was needed. This could be a consequence of *Galdieria* spp. slower O_2 production rates, ~120 $\mu mol_{O2}\ mg_{chlorophyll}^{-1}\ h^{-1}$(Mozaffari *et al.*, 2019), vs 710m $\mu mol_{O2}\ mg_{chlorophyll}^{-1}\ h^{-1}$ for *Synechococcus elongatus* PCC 7942 (Thomas, Bricker, & Klotz, 1993).

Table 2.2. Comparison of steady states values between axenic cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 during chemostat cultivation.

Culture[a]	***Methylacidiphilum* sp. RTK17.1**	**Coculture**	***Galdieria* sp. RTK37.1**
Chemostat conditions			
Dilution Rate (day^{-1})	0.167	0.167	0.278
Agitation (rpm)	250	250	400
Steady state values			
Concentration ($g_{DW}\ L^{-1}$)[c]	0.170 (± 0.006)	0.393 (± 0.014)	0.154 (± 0.002)
Biomass productivity (mg $L^{-1}\ h^{-1}$)[c]	1.18 (± 0.04)	2.74 (± 0.10)	1.78 (± 0.02)
Dissolved oxygen (mg L^{-1})[b]	0.267 (± 0.005)	0.388 (± 0.009)	0.977 (± 0.009)
CH_4 consumption[b]			
(mmol $L^{-1}\ h^{-1}$)	0.242 (± 0.08)	0.211 (± 0.014)	-
(mmol $h^{-1}\ g_{DW}^{-1}$)	1.42(± 0.05)	0.54 (± 0.04)	-
O_2 net production[b]			
(mmol $L^{-1}\ h^{-1}$)	-0.396 (± 0.032)	-0.214 (± 0.018)	0.149 (± 0.023)
(mmol $h^{-1}\ g_{DW}^{-1}$)	-2.33 (± 0.19)	-0.54 (± 0.05)	0.966 (± 0.152)
Biomass yield ($g_{DW}\ mol_{CH4}^{-1}$)[c]	4.89 (± 0.17)	13.0 (± 0.9)	-
CH_4 removal (%)[b]	40.5 (± 0.9)	34.3 (± 2.1)	-

[a]1 L stirred tank reactors with 900 mL working volume, 45 °C and pH 2.5. Feed gas was supplied continuously at a 16 mL min^{-1} rate. Feed gas composition for *Methylacidiphilum* sp. RTK17.1 and the coculture were: 2.1 % O_2, 1.1 % CH_4, 74 % CO_2, balance N_2, all in% v/v. For *Galdieria* sp. RTK37.1 feed gas composition was 3.0% O_2, 64% CO_2, rest N_2. Systems were considered at steady state when there was less than a 5 % OD_{600} change in a five day period. All chemostat cultures were N-replete. [b]12 day average after steady state was achieved. [c]Biomass concentration of accumulated one day outflow broth, with n = 3 technical replicates. Values in brackets represent one standard deviation.

In the coculture chemostat, a biomass concentration of 0.393 $g_{DW}\ L^{-1}$ and a biomass productivity of 2.74 mg $L^{-1}\ h^{-1}$ were achieved, both values greater than the respective axenic cultures values. The biomass yield on methane in the *Methylacidiphilum* reactor was 4.89 ± 0.17 $g_{DW}\ mol_{CH4}^{-1}$, which is close to the lower end of values typically reported for methanotrophs: 2.4-24.48 $g_{DW}\ mol_{CH4}^{-1}$ (Khoshnevisan *et al.*, 2019). In the coculture, the yield increased to 13.0 ± 0.9 $g_{DW}\ mol_{CH4}^{-1}$ due to the inflationary contribution of *Galdieria* biomass. However, the biomass yield and specific CH_4 consumption for *Methylacidiphilum* sp. RTK17.1 on chemostat are lesser than previously reported by Carere *et al.* (2017) (3.11 ± 0.27 $mmol_{CH4}\ h^{-1}\ g_{DW}^{-1}$ and 6.79 ± 0.55 $g_{DW}\ mol_{CH4}^{-1}$). In the coculture methane oxidation rate further slowed down, with only 34.3 % being removed, which represents a 0.54 ±0.04 mmol $h^{-1}\ g_{DW}^{-1}$ specific methane consumption. This is however a faster specific methane consumption than what was reported for the culture of *M. alcaliphilum* and *Synechococcus,* 0.234 mmol $h^{-1}\ g_{DW}^{-1}$(Hill *et al.*, 2017)[1]. The decreased *Methylacidiphilum* rates are a consequence of the agitation rates used to accommodate the microalgae, as I decided to use a 250 rpm agitation rate in coculture to avoid damaging the bigger *Galdieria*'s cells. This was later found not to be

[1] Calculated from Hill *et al* (2017) considering a CH_4 uptake of 0.535 Cmmol $g^{-1}h^{-1}$ for M. *alcaliphilum* in the coculture, and a mass fraction of 0.562 for Synechococcus and 0.438 for M. *alcaliphilum* in the coculture.

neccesary. To present a fair comparison I also decreased the agitation in the methanotroph reactor from 800 to 250 rpm. The change decreased OD_{600} from 0.92 to 0.29, and CH_4 removal from 87.2 to 40.5 %. For methanotroph reactors increasing methane mass transfer has a major effect on biomass concentration, yields, and growth rates. For example, increasing the gas transfer coefficient in U-Loop reactors by a factor of 2.5 increased methanotroph concentration 3-fold (Kerckhof *et al.*, 2021). Also, I have increased average growth rate of *Methylacidiphilum* sp. RTK17.1 in a 10 L STR reactor from 0.094 to 0.720 day^{-1} by increasing gauge pressure from 0 to 500 mbar (Table H.1).

The beneficial effect *Galdieria* exhibited on CH_4 oxidation in batch was not observed in chemostat culture. This is consistent with our hypothesis that CH_4 oxidation was enhanced in batch cocultures due to increased O_2 concentrations within the liquid phase. The specific CH_4 and O_2 rates for the methanotroph in the axenic chemostat are close to the ones exhibited by the bacterium in the batch cocultures, where O_2 was continuosly supplied. As these rates were shown to be sensitive to O_2 limitation, it follows that there was no O_2 limitation on the methanotrophic reactor, and even less on the coculture reactor as the microalgae would have produced extra O_2. As the *Methylacidiphilum* sp. RTK17.1 in coculture did not require additional O_2, it obtained no benefit from the oxygenic activity of *Galdieria* sp. RTK37.1. Also, the relative concentration of each microorganism could play a part in the decrease, as it has been established that the photoautotroph:heterotroph inoculum proportion is a key factor contributing to mixed growth performance with microalgae (Santos & Reis, 2014). This has also been observed for other types methanotroph cocultures, for example Jeong *et al.* (2014) showed that the bacterium *Sphingopyxis* sp. NM1 stimulates growth and CH_4 oxidation rates of *Methylocystis* sp. M6 when using a 1:9 M6:NM1 volumetric ratio; but not when the ratio was 9:1 or 1:1. It was found that *Sphingopyxis* enhanced transcriptional expression of genes involved in methane oxidation when it was more abundant than *Methylocystis*. A similar concentration dependant effect on methane oxidation was observed with mixed cultures of *Methylocystis* sp. M6 and *Hyphomicrobium* sp. NM3 (Jeong & Kim, 2018).

2.3.3 Nutritional Analysis

To assess the suitability of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 biomass as animal feeds, nutritional analysis of several cultures grown in batch (1 L gastight Bottles), static liquid with continuous gas flow (1 L STR, 10 L STR, and 40 L photobioreactor), or in chemostat (1 L STR reactors) was performed (Figure 2.5). Non-nitrogen limited cultures of *Methylacidiphilum*, *Galdieria* and cocultures exhibited broadly similar macromolecular compositions, with protein content ranging from 50 to 60 %, carbohydrates from 30 to 40 %, fats from 4 to 9 %, and ash ranging from 0.6 to 3.0 %. In comparison, the protein content of soybean meal is 44 % (w/w, crude protein) and fishmeal is 60 % (w/w, crude protein) (Elangovan & Shim, 2000). The *Methylacidiphilum* chemostat would require 5.88 kg of methane to produce 1 kg of crude protein, while the coculture chemostat would require 2.15 kg of methane. In contrast bacterial meal from *Methylococcus capsulatus* grown in a specially designed/optimised loop reactor requires 1.7 kg methane per kg of crude protein (Overland *et al.*, 2010).

Methanotroph cultures tend to be slightly richer in carbohydrates and poorer in fats, while the coculture tends to have compositions closely resembling the axenic alga when grown in similar conditions. This is

consistent with other cocultures of *Galdieria* sp., for example Zhu *et al.* (2022) found that *Galdieria sulphuraria* grown mixotrophically in ultrahigh-NH_4^+ wastewater from industrial effluents, showed no significant difference in macromolecular contents when cultured in sterile or non-sterile media. However, the nutritional profile of the axenic *G. sulphuraria* (proteins 42.6 to 50.8 %, lipids 21.9 to 39.1 %, and carbohydrates 5.96 to 6.02 %) do contrast with our findings for *Galdieria* sp. RTK37.1. This can be explained by the mixotrophic mode of culture, as in microalgae it has been shown to affect lipid, protein, and carbohydrate biosynthesis, with protein content being negatively correlated with lipid content (Kadkhodaei *et al.*, 2015). Heterotrophic cells of *Galdieria sulphuraria* and *Galdieria partita* have been found to be ~30% larger than autotrophically grown cells, and to have undergone important change to internal cell structure (Barone *et al.*, 2020; Stadnichuk *et al.*, 1998). Our findings also contrast with a reported consortium of *C. sorokiniana* and *M. capsulatus,* where the coculture displayed lower protein concentrations (28 % of the dry weight) than their respective axenic cultures (45 % and 53 % respectively), but higher fat contents (34 % for coculture, 30 % of the algae, and 22 % for the methanotroph) (Rasouli *et al.*, 2018).

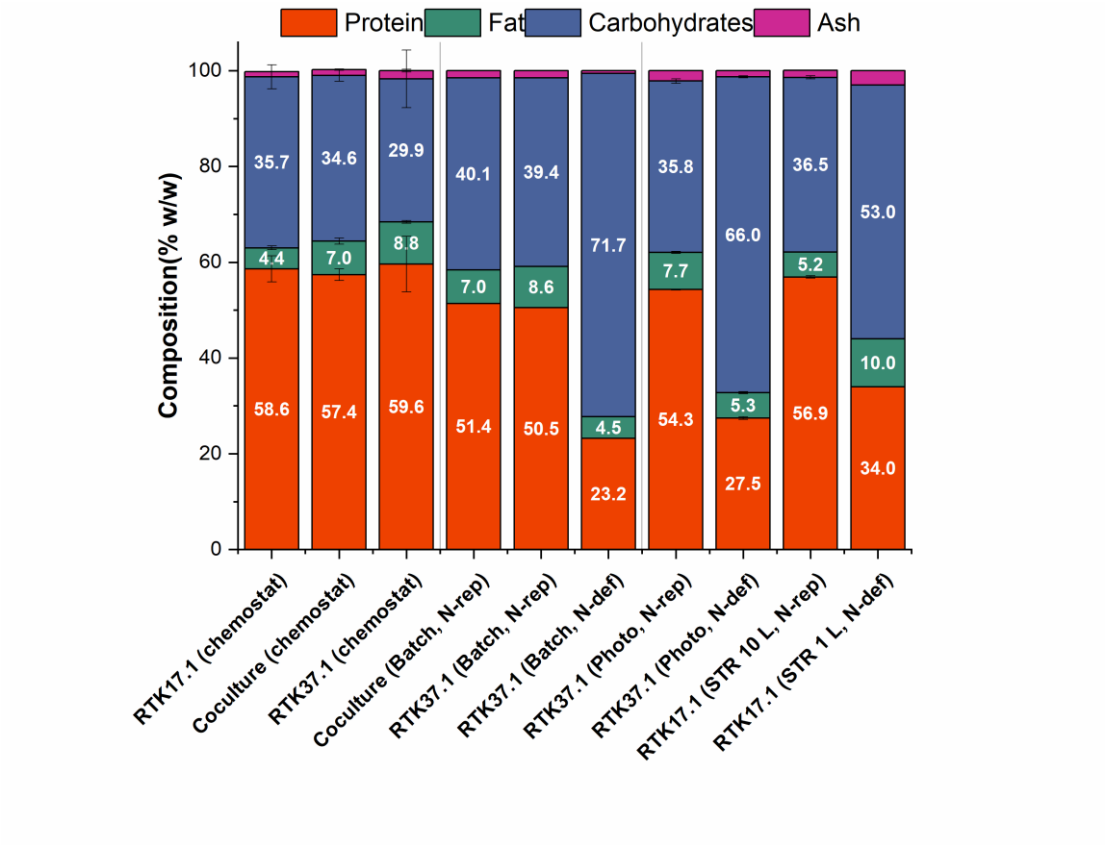


Figure 2.5. Macromolecular composition comparison between cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1. RTK17.1 refers to *Methylacidiphilum* sp. RTK17.1, and RTK37.1 to *Galdieria* sp. RTK37.1. Chemostat refers to steady state reactors with conditions as referred in Table 2.1. Batch refers to batch 1 L gastight bottles cultures. Photo refers to 80 L photobioreactors, and STR to stirred tank reactors, both with static liquid and continuous gas feed rate. N-rep refers to cultures harvested as soon as NH_4^+ was depleted, while N-def to cultures grown past NH_4^+ depletion. For bottle cultures, duplicates samples were pooled together, and for the 1 L STR reactor only 1 sample was processed. For the remaining samples error bars represent 1 standard deviation for technical replicates (n = 3), except for RTK37.1 (chemostat) where n = 2.

Galdieria sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1 grown for extended periods of ammonium depletion appreciably decrease their protein content and increase carbohydrate contents. This makes

sense as both microorganisms accumulate glycogen as a storage compound under nitrogen limitations (Carere *et al.*, 2019; Khadem, Pol, *et al.*, 2012; Sydney *et al.*, 2019). Additionally, for microalgae nitrogen is necessary for continuous protein synthesis and indirectly for pigment formation (Kadkhodaei *et al.*, 2015), and in N-starved cells, free amino acid synthesis is slowed down (Salbitani & Carfagna, 2020).

Table 2.3. Amino acid distribution for cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1.

Amino Acid ($g/100\ g_{DW}$)	**RTK17.1[a,c] (chemostat)**	**Coculture[c] (chemostat)**	**RTK37.1[b,d] (chemostat)**	**RTK17.1[a,e] (STR, N-rep)**	**RTK37.1[b,f] (photo, N-rep)**	**RTK37.1[b,g] (photo, N-def)**
Essential amino acids ($g/100\ g_{DW}$)[h]						
Histidine	1.12 (± 0.10)	0.82 (± 0.07)	0.77 (± 0.09)	1.25 (± 0.03)	0.54 (± 0.01)	0.29 (± 0.01)
Isoleucine	2.99 (± 0.01)	2.64 (± 0.10)	3.04 (± 0.37)	2.68 (± 0.04)	2.64 (± 0.03)	1.24 (± 0.01)
Leucine	4.82 (± 0.00)	3.99 (± 0.19)	4.41 (± 0.55)	4.54 (± 0.07)	3.80 (± 0.07)	1.70 (± 0.02)
Lysine	4.01 (± 0.17)	3.29 (± 0.22)	3.48 (± 0.39)	4.36 (± 0.09)	3.05 (± 0.08)	1.57 (± 0.01)
Methionine	1.59 (± 0.02)	1.27 (± 0.08)	1.35 (± 0.00)	1.62 (± 0.03)	1.33 (± 0.05)	0.57 (± 0.01)
Phenylalanine	3.66 (± 0.12)	2.68 (± 0.24)	2.71 (± 0.37)	2.67 (± 0.02)	2.25 (± 0.06)	1.12 (± 0.02)
Threonine	2.47 (± 0.05)	2.88 (± 0.07)	3.63 (± 0.45)	2.12 (± 0.04)	3.02 (± 0.08)	1.76 (± 0.02)
Tryptophan	NR[i]	NR[i]	NR[i]	1.40 (± 0.03)	0.82 (± 0.04)	0.42 (± 0.02)
Valine	3.20 (± 0.01)	3.25 (± 0.09)	3.84 (± 0.48)	2.83 (± 0.04)	3.34 (± 0.03)	1.86 (± 0.01)
Essential AA	23.87 (± 0.28)	20.84 (± 0.90)	23.23 (± 2.69)	23.47 (± 0.32)	20.79 (± 0.40)	10.52 (± 0.05)
Non essential amino acids ($g/100\ g_{DW}$)[h]						
Cysteine	0.54 (± 0.04)	0.99 (± 0.13)	1.34 (± 0.01)	0.59 (± 0.01)	1.06 (± 0.05)	0.69 (± 0.01)
Tyrosine	2.96 (± 0.03)	3.60 (± 0.06)	4.69 (± 0.62)	2.33 (± 0.04)	3.87 (± 0.06)	2.37 (± 0.02)
Glycine	3.14 (± 0.13)	2.31 (± 0.12)	2.36 (± 0.33)	2.67 (± 0.14)	2.09 (± 0.01)	0.96 (± 0.03)
Glutamic acid	6.69 (± 0.58)	7.04 (± 0.53)	8.08 (± 0.93)	7.84 (± 0.19)	6.68 (± 0.65)	3.92 (± 0.05)
Serine	2.49 (± 0.22)	3.12 (± 0.13)	4.04 (± 0.54)	2.13 (± 0.06)	3.29 (± 0.23)	1.76 (± 0.03)
Aspartic Acid	4.45 (± 0.19)	4.15 (± 0.24)	4.95 (± 0.59)	4.47 (± 0.04)	4.14 (± 0.19)	1.78 (± 0.05)
Arginine	2.77 (± 0.07)	2.75 (± 0.10)	3.40 (± 0.40)	2.80 (± 0.05)	3.11 (± 0.05)	1.26 (± 0.02)
Taurine	0.05 (± 0.00)	0.03 (± 0.00)	0.07 (± 0.00)	NR[i]	0.13 (± 0.01)	0.05 (± 0.01)
Proline	2.52 (± 0.04)	2.66 (± 0.12)	3.13 (± 0.35)	2.21 (± 0.03)	2.36 (± 0.05)	1.45 (± 0.01)
Alanine	3.22 (± 0.04)	2.92 (± 0.12)	3.42 (± 0.43)	2.95 (± 0.04)	2.83 (± 0.08)	1.26 (± 0.01)

[a] RTK17.1 stands for *Methylacidiphilum* sp. RTK17.1. [b] RTK37.1 stands for *Galdieria* sp. RTK37.1. [c]1 L stirred tank reactor, dilution rate 0.167 day^{-1}, and 250 rpm agitation rate. [d]1 L stirred tank reactor, dilution rate 0.278 day^{-1}, and 400 rpm agitation rate. [e]10 L stirred tank reactor static liquid and continuous gas feed rate, with intermittent NH_4^+ additions. [f]40 L tubular photobioreactor, static liquid and continuous gas feed rate, cultivated until NH_4^+ depletion. [g]40 L photobioreactor, static liquid and continuous gas feed rate, cultivated 7 days past NH_4^+ depletion. [h]Values in brackets are standard deviations with n = 3 technical replicates for all biomass samples, except for RTK37.1 (chemostat) with n = 2. [i]Amino acid not measured.

With respect to amino acid composition, glutamic acid was the most concentrated amino acid in all cultures (Table 2.3). In chemostat *Methylacidiphilum* sp. RTK17.1 is significantly richer than *Galdieria sp.* RTK37.1 in histidine (p-value < 0.05), methionine (p-value < 0.001), and phenylalanine (p-value < 0.05). Conversely, the microalgae displayed greater concentrations of threonine (p-value < 0.05), cysteine (p-value < 0.001), tyrosine (p-value < 0.05), and serine (p-value < 0.05). For the coculture, no amino acid concentration significantly varies from one of the axenic cultures (p-value > 0.05) except for tyrosine and cysteine. The coculture chemostat is more concentrated in tyrosine and cysteine that the axenic *Methylacidiphilum* (p-values < 0.001, and < 0.005 respectively), but less concentrated than *Galdieria* (p-value < 0.05 for both amino acids). The chemostat coculture had an essential amino acid concentration of 20.84 ± 0.90 g/100

g_{DW} which is greater than the values reported for *Methyloparacoccus murrelli* LMG 27482 in coculture with *Cupriavidus necator* LMG 1201 (14.5 g/100 g_{DW}) (Kerckhof *et al.*, 2021).

The FAO/WHO/UNU standard states the required daily intake of specific amino acids by humans (WHO/FAO/UNU Expert Consultation, 2007), and is often used in literature to evaluate if a protein source, consumed as the only dietary protein source, would provide all the required indispensable amino acids for an adult (at a recommended protein intake level of 0.66 $g_{protein}$ kg^{-1} day^{-1}). Soybean meal and fishmeal are common proteins used as animal feed (Kerckhof *et al.*, 2021). In Figure 2.6 I compare the indispensable amino acid profiles with soybean meal, fishmeal and the FAO/WHO/UNU standard. While there were differences on the protein and carbohydrate concentrations for each organism depending on their growth conditions, the amino acid profiles remain largely the same. It is often the case for both microalgae and methanotrophs biomass that growth conditions largely affect macromolecular composition, but not the amino acid profile (Batista *et al.*, 2013; Overland *et al.*, 2010; Templeton & Laurens, 2015). As an example, Templeton and Laurens (2015) found for *Chlorella sp.* that sampling at three different lifecycle stages, starting with nitrate-replete conditions and proceeding to nitrate deprivation, resulted in only minor changes to glutamine, alanine, lysine and arginine content. *Scenedesmus* sp., *Chlorella vulgaris*, and *Nannochloris* sp., similarly didn't show any important variation to protein quality at different growth stages. In my cultures, for *Methylacidiphilum* the largest difference was in the sum of phenylalanine and tyrosine (11.3 $g/g_{protein}$ in chemostat vs 8.8 $g/g_{protein}$ in a 10 L STR), and there was < 0.5% difference in the concentrations of valine and threonine. For *Galdieria,* leucine concentration went from 7.0 $g/g_{protein}$ in chemostat to 6.2 $g/g_{protein}$ in the ammonium depleted photobioreactor, and there was < 0.5% difference in the concentrations of histidine, cysteine, and methionine between reactor systems. The proteins from *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1, and the cocultures were comparable in quality to both fishmeal and soybean meal (Figure 2.6). The methanotroph protein meets all the FAO/WHO/UNU reference protein concentrations, it also surpasses all indispensable amino acid concentrations of soybean meal and fishmeal, with the exceptions of cysteine in soybean meal, and histidine in both reference proteins. The microalgae and coculture proteins are marginally deficient in histidine when compared to the FAO/WHO/UNU reference protein, have lesser lysine and methionine concentrations than fishmeal but otherwise are comparable (or superior) regarding indispensable amino acid content.

Lysine is the main limiting amino acid for animal species fed grains as a main energy source (Erdman *et al.*, 1977; Skrede *et al.*, 1998); with most plant-based proteins displaying relatively poor contents of lysine (3.6 ± 0.6%) and methionine (3.6 ± 0.6%) (Gorissen *et al.*, 2018). This makes the proteins from *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 attractive as replacement feedstocks, as they have higher quantities of methionine and cysteine (compared to soybean meal and fishmeal), and the methanotroph lysine content is comparable to fishmeal and higher than soybean meal. In comparison lysine has been reported to be deficient in bacterial protein meals produced by a consortium of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans*, *Bacillus brevis*, and *Bacillus firmus* grown on natural gas (Skrede *et al.*, 1998). Lysine is also limiting in most studied cocultures of methanotrophs with hydrogen oxidizing bacteria, including *Methyloparacoccus murrelli* LMG 27482 with *Cupriavidus necator* (Kerckhof *et al.*, 2021). Methionine has been found to be in deficient quantities in most analysed microalgae (Roy & Pal,

2015), and in commercial *Spirulina* (Nyyssölä *et al.*, 2022). A community of methanotrophs dominated by *Methylomonas* sp. and *Methylocystis* sp. have been found lacking in both lysine and methionine concentrations (Valverde-Pérez *et al.*, 2020; Zha *et al.*, 2021). Kerckhof *et al.* (2021) calculated that the amount of microbial biomass needed to meet the individual amino acid requirements of a 62 kg human adult ranged from 139 g, for a coculture of *Methyloparacoccus murreli* with *Cupriavidus necator* (lysine being the limiting amino acid) to 1982 g for an axenic culture of *Hydrogenophaga electrium* (limited by isoleucine). Lysine and isoleucine were the most common limiting amino acids in Kerckhof *et al.* (2021) cultures. For our chemostat cultures, a similar calculation reveals that histidine is the limiting amino acid for all cultures, and the biomass required to meet the required amino acids consumption in humans, would be 568 g for *Methylacidiphilum*, 804.8 g *Galdieria*, and coculture 753.7 g for the coculture.

2.4 Conclusions

This study demonstrates a 'proof-of-concept' that *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 can be successfully grown together in coculture via batch and continuous operation. The methanotroph benefits from the interaction when in a low O_2 environment, as seen by the increased growth and methane oxidation rates in serum bottle experiments. I also found, at the bacterium:alga proportions investigated, no evidence of *Galdieria* sp. RTK37.1 being negatively affected by the presence of *Methylacidiphilum* sp. RTK17.1. However, increasing the relative concentration of methanotroph could introduce a shading effect, which could limit photosynthesis, and in consequence coculture growth. This could also be the case for higher concentration broths in general, as shading effects and higher oxygen demands could further limit O_2 availability. It is clear then that O_2 plays a fundamental role on *Methylacidiphilum-Galdieria* interactions, but to study it further, a method to rapidly quantify the relative abundance of each microorganism, that does not rely on gas uptake rate, is required. According to their amino acid profiles, *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 each exhibit a similar nutritional quality to soybean meal and fishmeal and could potentially be used as feed for animals.

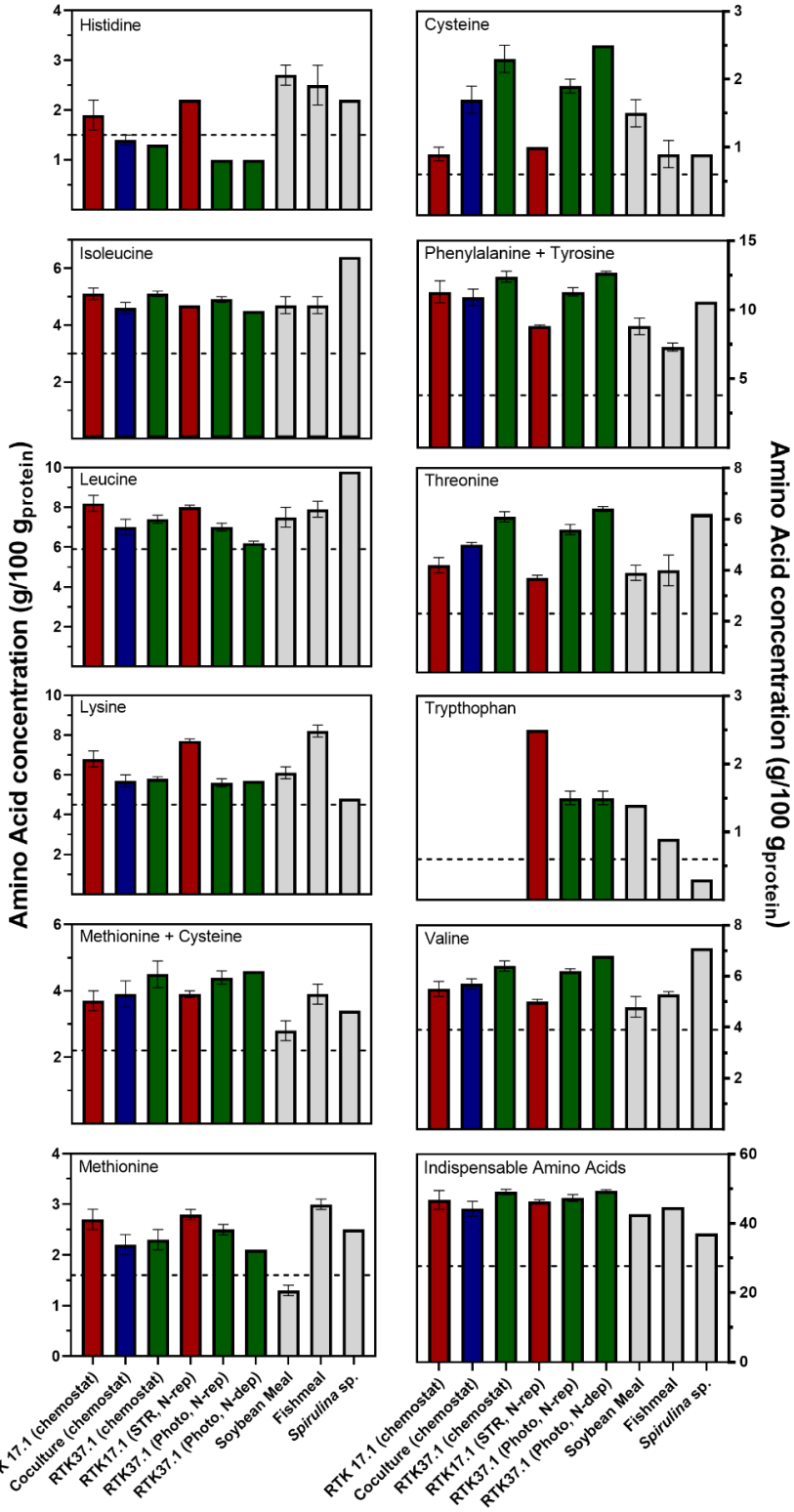


Figure 2.6. Indispensable amino acid composition comparison between cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1, and reference proteins. Error bars represent standard deviations with n = 3 technical replicates for all biomass samples, except for RTK37.1 (chemostat) with n = 2. Bars are colour coded red for *Methylacidiphilum* sp. RTK17.1, green for *Galdieria* sp. RTK37.1, blue for cocultures, and grey for reference proteins. The dashed black line represents the FAO/WHO/UNU reference protein. Reference proteins values for soybean meal and fishmeal were taken from Overland *et al* (2010), and *Spirulina* from Becker (2007). Reactor systems as explained in Table 2.3.

Chapter 3. Determination of relative abundance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 with a combination of differential sedimentation, optical density, and fluorescence

Abstract

Oxygen is fundamental to *Methylacidiphilum-Galdieria* coculture interactions, but to study coculture dynamics further, a method to rapidly quantify the relative abundance of each microorganism (methanotroph-algae) is required. Existing methods are often time consuming or require expensive dedicated equipment (Helmi *et al.*, 2014; Imashiro *et al.*, 2020; Van Nevel *et al.*, 2017). However, several simple laboratory techniques can be used to exploit differences in each microorganism's physical characteristics (e.g. cell size, shape, density, fluorescence) to quantify their relative concentration. In this study, I report the development and validation of a novel method based on differential sedimentation, optical density and autofluorescence (DSOF method). The DSOF method uses differences in settling velocities and autofluorescence at 670 nm emission (590 nm excitation) of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 to determine the relative concentration of each in a coculture. A coculture sample is centrifuged for a short duration under a weak centrifugal force. Supernatant fluorescence and optical density values are then compared to the non-centrifuged coculture sample. With these four values, the biomass concentration of both *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in the coculture can be derived. To test the validity of the DSOF method, artificial non-growing *Methylacidiphilum-Galdieria* suspensions were prepared from axenic stocks across a wide range of pre-defined biomass concentrations (OD_{600}). The absolute error of derived biomass concentration values using the DSOF method were determined to be ≤ ±0.1 A.U. if [*Galdieria*] ≤ 2.0 A.U., and [*Methylacidiphilum*] ≤ 1.5 A.U. Finally, the DSOF method was evaluated using active cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 under conditions which: 1) suppressed methanotroph growth (no CH_4), 2) supressed microalgae growth (no light), or 3) permitted growth of both microorganisms (with CH_4 and light). Derived *Methylacidiphilum-Galdieria* concentrations were consistent with their expected growth behaviour and my prior observations. I conclude that the DSOF method is a simple, rapid, and accurate method to quantify the relative concentration of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture. The method does not rely on headspace gas measurements, requires small liquid volume samples (2-3 mL), and is likely applicable to other methanotroph-algae cocultures.

3.1 Introduction

I concluded in Chapter 2 that oxygen (O_2) is vital to *Methylacidiphilum-Galdieria* coculture interactions and highlighted a need for a rapid method to quantify the relative abundance of each microorganism in coculture. Investigations into methanotroph-photoautotroph coculture dynamics often report total biomass concentrations (as either optical density or dry biomass) without differentiating the relative proportion of each microorganism (Hill *et al.*, 2017; Rasouli *et al.*, 2018; Yan *et al.*, 2022). Where the relative abundance is reported, direct cell counting via flow cytometry is typically used (Hill *et al.*, 2017; Ruiz-Ruiz *et al.*, 2020). A combination of several techniques is sometimes used to quantify coculture dynamics. As an example, Kerckhof *et al.* (2021) used flow cytometry in concert with selective plate counting to quantify the relative proportion of methanotrophs-hydrogenotrophs in coculture. As a further example, a combination of

gravimetric methods (to determine total coculture biomass), flow cytometry (to track *Galdieria* cell density), and Illumina HiSeq DNA sequencing (to evaluate bacterial variation) has previously been used by Zhu *et al.* (2022) in mixed cultures of *Galdieria sulphuraria* and bacteria within ammonium-rich wastewater.

Cell counting via flow cytometry is typically fast, has less than 5 % of instrumentation error, and can detect as few as 100 cell mL^{-1} (Hammes & Egli, 2010). However, it requires dedicated expensive equipment and highly trained technical support (Helmi *et al.*, 2014; Van Nevel *et al.*, 2017). In addition, cell fixation is sometimes required for preservation of samples or to make cells permeable to dyes (for staining) (Günther *et al.*, 2008). If sample fixation protocols are not optimized, large error can be obtained due to sticking of cells and nonuniform distribution in samples (Badr *et al.*, 2020). Alternatively, cell counting can be performed using hemocytometry, however the process is tedious, labour intensive, and error varies wildly with skill of the user (Imashiro *et al.*, 2020; Van Nevel *et al.*, 2017). Additionally, calculating biomass concentrations using cell counts can be problematic as biomass accumulation can occur in the absence of cell division. For example, under nitrogen depletion *Methylacidiphilum spp.* are known to slow cell division but will continue to accumulate mass via glycogen accumulation (Carere *et al.*, 2019; Khadem, van Teeseling, *et al.*, 2012). Similarly Type II methanotrophs, like *Methylosinus trichosporium* OB3p and *Methylocystis parvus* OBBP, synthesise polyhydroxybutyrate (PHB) granules when at least a growth factor is absent but CH_4 and O_2 are available (Rostkowski *et al.*, 2013). Phototrophs are similarly known to slow rates of (or cease) cell division and accumulate intracellular polymers in response to nitrogen starvation (Sinetova *et al.*, 2006).

Badr *et al.* (2020) recently reported a computational (E-C) method, based on the overall mass balance and individual growth stoichiometry coefficients, to quantify the proportional biomass of individual members in methanotroph-photoautotroph cocultures. The E-C method requires measurement of total optical density (at a wavelength dependant on the methanotroph-photoautotroph pair), headspace gas composition (CH_4, CO_2 and O_2), and dissolved CO_2 concentrations. It was verified with two model cocultures with good results: *Methylomicrobium alcaliphilum* 20ZR with *Synechococcus* sp. PC7002 and *Methylococcus capsulatus* with *Chlorella sorokiniana*. Unfortunately, a major drawback of the E-C method is its reliance on gas and dissolved carbon dioxide (CO_2) concentrations. For small volume batch experiments, continuously monitoring the gas phase is impractical; as headspace samplings are taken, gases could be removed (without replacement) in such a way as to impact experimental conditions**.** Additionally, the authors recognize that changes to headspace pressure or gas flow rates could cause significant errors if not accounted for. As total gas production and consumption are not necessarily balanced, this would often be the case for several cocultures. Using the sampling method described by Stone *et al.* (2019) was offered as a solution, however this method involves injecting an inert gas (e.g. Ar) to account for pressure losses in batch, or as a tracer gas for continuous systems. Thus, it: 1) modifies the headspace composition, which might not be desired, 2) may interfere with GC measurements (as Ar, N_2, and He are often used as carrier gases); and 3) does not solve the problem if the cocultures produce more gas than what they consume. The E-C method also assumes stoichiometric coefficients are readily available and constant regardless of axenic or coculture growth, which might not be the case. For example, in cocultures of *Scenedesmus obliquus* with the methanotroph *Methylocystis bryophila*, the yield of biomass on CH_4 changed from 0.18 to 0.30 mol_C/mol_{CH4} as O_2 headspace concentration increased from 10 to 50 % v/v (Li *et al.*, 2022). At low O_2

concentrations the microalga tended to grow autotrophically and the methanotroph would convert CH_4 into CO_2, while at high O_2 concentrations, heterotrophic algal growth would dominate, the methanotroph would not grow and release organic matter instead of CO_2. Thus, synergistic (or inhibitory) interactions in cocultures could modify stoichiometric coefficients and interfere with the E-C method. This could also be the case for *Methylacidiphilum* spp. as its metabolic flexibility suggests the possibility of it using metabolites produced by other species (e.g. methanol, H_2/CO_2, and formic acid) which would modify the yields of biomass on CH_4 (Carere *et al.*, 2017; Carere *et al.*, 2021; Carere *et al.*, 2019; Pol *et al.*, 2007). Additionally, *Methylacidiphilum* spp. produces biomass via CO_2 fixation using the Calvin-Benson-Bassham cycle (Khadem *et al.*, 2011), which would generate an additional CO_2 sink not accounted for by the E-C method.

By combining common, rapid, and inexpensive laboratory techniques (e.g. spectrophotometry, centrifugation and fluorescence measurement), the physical characteristics of microorganisms (e.g. cell size, shape, density, fluorescence) can be used to derive the relative proportion of members in coculture. Optical density (OD) is commonly used to measure biomass concentrations of axenic cultures by preparing a calibration curve that correlates cell concentration with absorbance readings at a specified wavelength (Stone *et al.*, 2017). Absorbance at specific wavelengths has been previously used to separately quantify both *Galdieria* (e.g. 800 and 750 nm) (Graverholt & Eriksen, 2007; Henkanatte-Gedera *et al.*, 2017; Hirooka & Miyagishima, 2016) and *Methylacidiphilum spp*. (e.g. 600 nm) (Awala *et al.*, 2021; Carere *et al.*, 2017; Khadem, van Teeseling, *et al.*, 2012). This approach fails for cocultures, however, as an OD reading at a single wavelength does not correspond to a unique concentration profile (Stone *et al.*, 2017).

Microalgae use pigments to absorb light for photosynthesis (i.e. chlorophyl, phycocyanin, and carotenoids) (Baer, Heining, Schwerna, Buchholz, & Hübner, 2016). These photosynthetic pigments are known to show autofluorescence, so *in vivo* fluorescence measurement is often used for the estimation of chlorophyll and other pigments' concentrations (Gregor & Maršálek, 2004). For example, phycocyanin, a pigment produced by *Galdieria* spp., exhibits fluorescence at 670 nm when excited at 590 nm (Gregor & Maršálek, 2005). Pigment concentration can be used to provide estimates of total photosynthetic biomass (Stewart & Farmer, 1984), however the method can be unreliable to track microalgae growth during culture, as pigment content in cells is greatly influenced by physiological state, age of cells, growth conditions, and light intensity/quality (Baer *et al.*, 2016; Schagerl, Siedler, Konopáčová, & Ali, 2022). Thus, autofluorescence measurement, alone or in combination with OD, is not enough to specifically parse out microalgae abundance in coculture.

Microalgae cells are typically much larger than methanotroph cells (Mata, Martins, & Caetane, 2012; Reis, Thottathil, & Prairie, 2022), hence their rate of sedimentation can be used to partially separate them. *Galdieria sulphuraria* cell diameter is ~ 3 - 9 µm (Vítová *et al.*, 2016), photoautotrophically grown cells are usually oval-shaped with average size of 3.8 x 5.0 µm (Sinetova *et al.*, 2006; Stadnichuk *et al.*, 1998). In contrast, *Methylacidiphilum kamchatkense* Kam1 is rod shaped with a length (l) of 0.8–1.0 µm and a diameter (d) of 0.45–0.65 µm (Islam *et al.*, 2008). Other described verrucomicrobial methanotrophs are similarly sized; l: 0.8–2.0 µm x d: 0.40–0.65 µm (Op den Camp *et al.*, 2009). According to Stokes Law, sedimentation/centrifugation velocities are proportional to the square of the particle diameter (Salim, Gilissen, Rinzema, Vermuë, & Wijffels, 2013), *Galdieria* cells should therefore settle at a rate ~100-fold faster than *Methylacidiphilum*. It follows that a short duration centrifuge under weak centrifugal forces (<

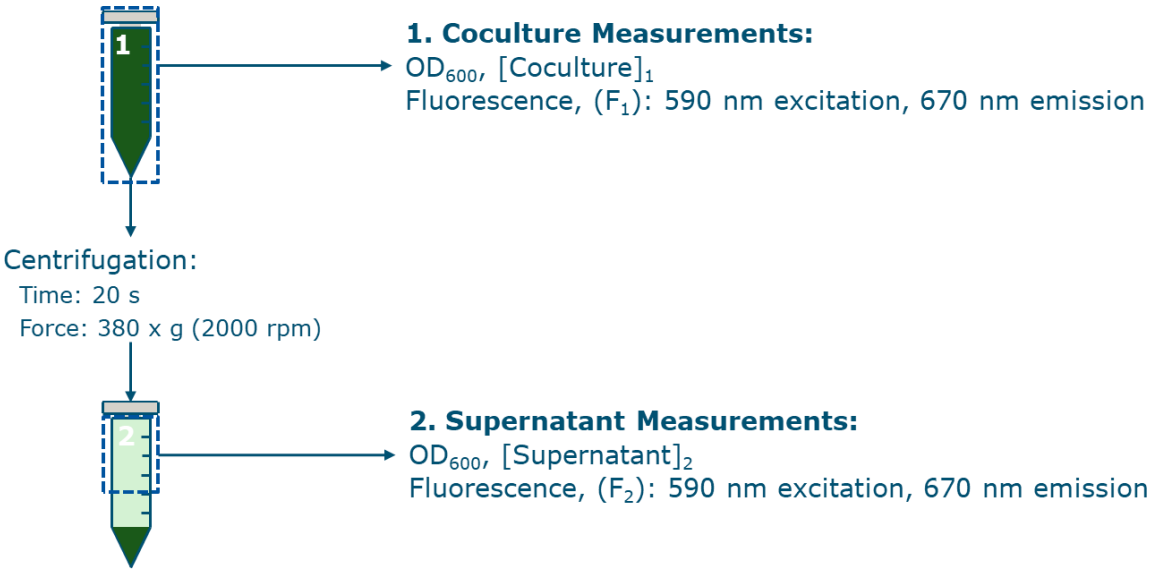
400 G) could potentially separate a fraction of microalgae from coculture, without significant bacterial sedimentation. Photopigments concentrations (e.g. chlorophyl, phycocyanin) are linked to photoautotroph concentrations (Stewart & Farmer, 1984), and *in vivo* fluorescence can be used for the estimation of pigment concentration (Gregor & Maršálek, 2005). Thus, by measuring the reduction in fluorescence and optical density in the supernatant, I quantified how much of the algae separated, and by extension how much was present in the original sample.

In this Chapter I develop and validate a method to measure relative abundance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture using a combination of differential sedimentation, optical density, and fluorescence techniques (DSOF method). Results show the DSOF method is able to rapidly quantify the abundance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture across a range of biomass concentrations with an acceptable error.

3.2 Materials and Methods

3.2.1 Theoretical framework of the Differential Sedimentation Optical Density Fluorescence (DSOF) method

The DSOF method exploits differences in the settling velocities and autofluorescence of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 to determine the relative concentration of each microorganism in coculture. A small volume of coculture is harvested and centrifuged for a brief time under weak centrifugal forces. Supernatant fluorescence and optical density are then compared to the original uncentrifuged sample. With these four values, the optical density (and hence biomass concentration) of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in the original coculture can be calculated. A graphical summary of the method is shown in Figure 3.1.



$$[Galdieria]_1 = \frac{[Coculture]_1 - [Supernatant]_2}{1 - {F_2}/{F_1}} \qquad [Methylacidiphilum]_1 = [Coculture]_1 - [Galdieria]_1$$

Figure 3.1 Graphical summary of the differential sedimentation optical density/fluorescence (DSOF) method.

Optical density is often used as a rapid method to measure the concentration of suspended microalgae and bacteria in solution; with absorbance at different wavelengths being used for both *Galdieria* (Graverholt & Eriksen, 2007; Henkanatte-Gedera *et al.*, 2017; Hirooka & Miyagishima, 2016) and *Methylacidiphilum spp*. (Awala *et al.*, 2021; Carere *et al.*, 2017; Khadem, van Teeseling, *et al.*, 2012). The DSOF method assumes that at 600 nm, absorbance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 is additive (Appendix C.1), so for any given coculture sample:

Supposition 1: OD_{600} values are additive for *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1, then:

$$[Methylacidiphilum]_1 + [Galdieria]_1 = [Coculture]_1 \quad (3.1)$$

Where subscript "1" denotes measurements made to the uncentrifuged coculture sample. Due to differences in cell size, settling velocities for *Galdieria* sp. RTK37.1 are much faster than for *Methylacidiphilum.* Therefore, if a coculture is centrifuged for a short duration under a weak enough centrifugal force (G), we would expect microalgae cells to settle predominantly over methanotrophic cells. As cells settle, the optical density in the coculture would decrease, so for the supernatant after centrifugation:

$$[Methylacidiphilum]_2 + [Galdieria]_2 = [Supernatant]_2 \quad (3.2)$$

Where subscript "2" refers to measurements made to the supernatant resulting from coculture centrifugation. If a coculture sample is centrifuged for a short period under a sufficiently weak centrifugation force, methanotroph cells will remain in suspension with minimal loss due to settling. My second assumption for the method is that there is no significant settling of methanotrophic cells:

Supposition 2: There is no significant settling of methanotrophic cells (Appendix C.2), then:

$$[Methylacidiphilum]_1 = [Methylacidiphilum]_2 \quad (3.3)$$

Galdieria cells are partly removed by centrifugation, so in the supernatant only a fraction of microalgae cells, "*y*" remain, then:

$$[Galdieria]_2 = y \cdot [Galdieria]_1 \quad (3.4)$$

The photosynthetic pigments of microalgae are known to show auto fluorescence (Gregor & Maršálek, 2004). Phycocyanin, a pigment produced by *Galdieria* spp., exhibits fluorescence at 670 nm when excited at 590 nm (Gregor & Maršálek, 2005). If we assume *Methylacidiphilum* sp. RTK17.1 shows no fluorescence (Appendix C.3), and the fluorescence of phycocyanin to be proportional to the microalgal cells remaining in suspension (Appendix C.3), which in turn is proportional to the optical density, then by measuring fluorescence of the original coculture (F_1) and the supernatant (F_2), we can approximate *y* as follows:

Supposition 3: The fraction of microalgae remaining in the supernatant can be approximated by the proportion of fluorescence of supernatant and original coculture:

$$y = \frac{[Galdieria]_2}{[Galdieria]_1} = {}^{F_2}/_{F_1} \quad (3.5)$$

We can now substitute Equations (3.4) and (3.3) in (3.2) and obtain the system of equations:

$$[Methylacidiphilum]_1 + [Galdieria]_1 = [Coculture]_1 \quad (3.1)$$
$$[Methylacidiphilum]_1 + y \cdot [Galdieria]_1 = [Supernatant]_2 \quad (3.6)$$

Thus, we can solve for $[Galdieria]_1$:

$$[Galdieria]_1 = \frac{[Coculture]_1 - [Supernatant]_2}{1 - y} \quad (3.7)$$

And for $[Methylacidiphilum]_1$:

$$[Methylacidiphilum]_1 = [Coculture]_1 - \frac{[Coculture]_1 - [Supernatant]_2}{1 - y} \quad (3.8)$$

3.2.2 Technical Description of the DSOF method

To differentiate the contribution of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 to the coculture OD_{600}, a 0.75 mL sample of coculture was harvested and the optical density, $[Coculture]_1$, was measured using an Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom). If sample OD_{600} was greater than 0.6, it was diluted and remeasured. Next, 200 µL coculture were transferred into a black, flat bottom (chimney well) 96-well microplate (Greiner Bio-One, Austria), and the fluorescence emission, F_1, at 670 nm (590 nm excitation), was measured using a microplate reader (Varioskan Lux,Thermo Scientific). Following this, a 1.5 mL coculture sample was transferred into a 1.5 mL centrifuge tube and centrifuged at 380 x g for 20 seconds in a microcentrifuge (Biofuge Pico, Heraeus Instruments). The resulting supernatant (0.75 mL) was then collected, and the OD_{600}, $[Supernatant]_2$, and fluorescence (F_2) were measured as described for the coculture above. Finally, the OD_{600} values for *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1 in coculture were then calculated using Equations 3.7 and 3.8 respectively.

3.2.3 DSOF Validation in artificial suspensions

To test the DSOF method, artificial “coculture” suspensions were prepared from axenic stock solutions across a range of predefined OD_{600} values. Aliquots of each, *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1, were diluted into sterile V4 media to the desired final concentration. For the methanotroph, *Methylacidiphilum* sp. RTK17.1 cells were harvested from a 1 L bioreactor (BioFlo 110; New Brunswick Scientific, Edison, NJ, USA) grown in chemostat (D = 0.278 day^{-1}) on a gas mixture of 67 % CO_2, 1.0 % CH_4, 2.1 % O_2 (balance N_2, all v/v) supplied at 16 mL min^{-1}. Temperature was kept at 45 °C, agitation at 400 rpm, and pH 2.5 (not controlled). Cells were stored at 4 °C until required, and then centrifuged at 5000 x g for 15 minutes before being resuspended with sufficient V4 media to achieve an OD_{600} of 4.0 A.U. For the microalgae stock, 300 mL V4 media (pH 2.5) was inoculated with *Galdieria* sp. RTK37.1 to an initial OD_{600} of 1.0 A.U. within 1 L Duran Pressure Plus Bottles equipped with bromobutyl rubber stoppers. The bottles were then subjected to vacuum for 3 minutes, and re-pressurized to 5 psi with an 80 % CO_2 and 20 % N_2 gas mixture. The bottles were incubated in a shaker at 150 rpm and 45 °C. Light was provided via

warm white LED strips at an intensity of 40 µmol photons m^{-2} s^{-1} measured at the bottle's wall. Cells were harvested at OD_{600} = 3.5 A.U. to ensure cultures were never nitrogen limited.

Several artificial coculture groups were prepared and analysed with the DSOF method in order to determine the method's precision and accuracy. For each group, one of the microorganism's OD_{600} was kept constant, while the other was varied in defined increments. In total 8 groups were analysed, 5 where the microalgae OD_{600} was constant ([*Galdieria*]$_K$: 0.28, 0.52, 1.00, 1.81, and 3.23 A.U.), and 3 where the methanotroph OD_{600} was constant ([*Methylacidiphilum*]$_K$: 0.44, 0.90, and 1.54 A.U.). Triplicates of each artificial mixture were analysed.

3.2.4 DSOF Validation in live batch cocultures

To evaluate the DSOF method on live cultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1**,** batch cocultures were performed under one of three conditions: 1) light, no CH_4 – to promote microalgae growth and suppress methanotroph growth, 2) no light, added CH_4 - to supress microalgae growth and promote methanotroph growth, or 3) light, added CH_4 – to promote growth of both microorganisms. For these experiments, the previously described stocks of microalgae and methanotroph were used to inoculate 1L Duran Pressure Plus Bottles, equipped with bromobutyl rubber stoppers, with 250 mL of broth in V4 media (pH 2.5). The supressed cultures had a starting *Methylacidiphilum* sp. RTK17.1 OD_{600} value of 0.45 A.U., and a starting *Galdieria* sp. RTK37.1 value of 0.45 A.U. The non-supressed cultures had initial OD_{600} values of 0.3 A.U. for *Methylacidiphilum* sp. RTK17.1, and 1.0 A.U. for *Galdieria* sp. RTK37.1. To promote methanotroph and supress microalgal growth, bottles were covered with aluminium foil, sealed, and then 100 mL of CH_4 and 60 mL of CO_2 were injected. To promote microalgal growth and supress methanotroph growth, bottles were injected with 160 mL of CO_2, no CH_4, and supplied with 40 µmol photons m^{-2} s^{-1} warm white LED lighting (i.e. no foil covering). To promote growth of both methanotroph and microalgae, bottles were injected with 100 mL of CO_2, 60 mL of CH_4, and illuminated as above. Table 3.1 shows the initial headspace gas concentrations for all conditions. The bottles were then cultivated on a shaking incubator (WiseCube WIS-10, Wisd Laboratory Instruments, Germany) at 45 °C, and 150 rpm. Each condition was run in triplicates.

Table 3.1. Initial CO_2, CH_4, and O_2 headspace gas concentrations for DSOF validation in live batch coculture experiments.

Desired Condition	Headspace concentrations[a] (% v/v)		
	O_2	CH_4	CO_2
1. Methylacidiphilum growth supressed	14.32 ± 0.01	0	17.29 ± 0.23
2. Galdieria growth supressed	14.27 ± 0.05	10.00 ± 0.04	6.55 ± 0.07
3. Galdieria and *Methylacidiphilum* growth allowed	15.06 ± 0.09	6.94 ± 0.15	10.60 ± 0.04

[a] Balance N_2 for all cultures.

To measure the microorganism's concentration, 2 mL liquid samples were harvested daily and analysed using the DSOF method. To ensure that CO_2/CH_4/O_2 concentrations weren't limiting growth, 20 mL headspace gas samples were analysed daily using a 490 micro-GC equipped with a thermal conductivity detector (Agilent Technologies, United States). To replenish gases to their initial concentrations, after

sampling, the bottles were opened on a Laminar Flow cabinet, left to equalize for 30 minutes, and re-gassed as previously described to the concentrations shown in Table 3.1. Growth was stopped after 6 days of culture or when total OD_{600} surpassed 3.5 A.U. Growth rates were calculated by fitting the appropriate concentration data to the exponential growth equation model in Prism Graphpad 9.4.1 ($Y = Y_0 e^{\mu_{max} t}$, where Y is the concentration in A.U., Y_0 is the concentration at time zero in A.U., μ_{max} is the specific growth rate in h^{-1}, and t is time in h).

3.3 Results and discussion

3.3.1 DSOF in artificial suspensions

In this Chapter a method to measure relative abundance of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture was developed using a combination of differential sedimentation, optical density, and fluorescence techniques (DSOF). To test the DSOF method, artificial suspensions were prepared from axenic stock solutions with known OD_{600}. For each group of samples, one of the microorganism's OD_{600} was kept constant, while the other was incrementally varied. In total 7 groups were analysed, 5 where the microalgae OD_{600} was constant ([*Galdieria*]$_K$ = 0.28, 0.52, 1.00, 1.81, and 3.23 A.U.), and 3 where the methanotroph OD_{600} was constant ([*Methylacidiphilum*]$_K$ = 0.44, 0.90, and 1.54 A.U.). In Figure 3.2 I show the results of the derived concentration values as a function of the methanotroph ([*Methylacidiphilum*]$_D$) and algae [*Galdieria*]$_D$ concentrations, for the samples where *Galdieria* OD_{600} was kept constant along each group. To visualise all the data on the same graph, the known (subscript K) *Methylacidiphilum* concentrations ([*Methylacidiphilum*]$_K$) were normalised by dividing them by the corresponding known *Galdieria* concentrations ([*Galdieria*]$_K$). For each data group, a linear regression was performed (not shown) using the derived concentrations (subscript D), and I show the limit of the 95% confidence prediction bands as dashed lines of the same colour. The known concentrations are shown as continuous lines. In Figure 3.2a the slopes of the derived concentration regressions should be equal to the slope of known concentration lines (as $y = mx$, with m = [*Galdieria*]$_K$). A two-tailed paired t-test revealed no significant difference (p-value > 0.05) between both set of slopes, this means that the expected *Methylacidiphilum* sp. RTK17.1 'derived' concentrations from the DSOF method are not significantly different from the 'known' values in the artificial suspensions. Likewise, in Figure 3.2b the intercepts of derived concentration regressions and the known concentration lines show no significance difference (p-value > 0.05). There is a good agreement between the derived values from the DSOF method, and the known OD_{600} values of each microorganism in the cocultures.

To further evaluate the precision and accuracy of the DSOF method for the constant *Galdieria* concentration groups, I show the relative and absolute errors of the derived concentrations in Figure 3.3. For [*Galdieria*]$_K$ ≤ 1.00 A.U., the errors for both [*Galdieria*]$_D$ and [*Methylacidiphilum*]$_D$ concentrations are < 10%, and absolute errors are within ± 0.1 A.U. However, as [*Galdieria*]$_K$ concentrations increase, so does the relative and absolute error for derived values. When [*Galdieria*]$_K$ > 1.00 A.U. both errors and standard deviations increase, with the greatest occurring where *Methylacidiphilum* concentrations < 0.5 A.U. at [*Galdieria*]$_K$ OD_{600} = 3.23 A.U. Nevertheless, even at the greater concentrations, most relative errors are < 20%, and most absolute errors are between ± 0.2 A.U. These observations are consistent with Stokes Law ideal suspension behaviour, in which particles sediment without interacting with other particles when in dilute

concentration (Quispe, Concha, & Toledo, 2000). As particle concentration increases, however, collisions become more frequent, and the relative rapidly settling large microalgae cells are more likely to encounter methanotroph cells. It's possible these collisions could result in methanotroph-cell attachment to the algae, which would effectively remove them from the supernatant. The attachment could occur by a mechanism akin to bioflocculation, wherein the presence of bacteria can induce rapid formation and growth of flocs in bacteria-microalgae cocultures (Ramanan, Kim, Cho, Oh, & Kim, 2016). The flocs are groups of cells of both microorganisms that come in contact and become attached (Powell & Hill, 2013). Exopolysaccharides (EPS) excreted by bacteria or microalgae can serve as bioflocculants (Babiak & Krzemińska, 2021), i.e. substances that neutralize the negatively charged cell surface, which then negate the electrostatic repulsive forces that keep cells apart. *Galdieria sulphuraria* has been reported to produce up to 115.8 mg L^{-1}_{Broth} of EPS (Zhu *et al.*, 2022). There are no reports of EPS production in *Methylacidiphilum* spp., but other methanotrophs like *Methylomicrobium alcaliphilum* 20z have been found to produce up to 2.64 g L^{-1}_{Broth} of EPS (Cantera, Sanchez-Andrea, *et al.*, 2018). Also van der Ha *et al.* (2011) reported floc formation on a methane oxidizing community from anaerobic sludge tanks when cocultured with a microalgae community dominated by *Scenedesmus* sp**.** So, it is possible that there is EPS production in *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 cocultures. The birth of flocs (formation by collision of two particles) is dependent on cell concentration (Salim *et al.*, 2013), and the efficiency of aggregation depends on the bacteria to algae ratio (Powell & Hill, 2013). Thus, increased *Galdieria* concentrations could lead to increased error. A similar effect was reported when using flow cytometry to quantify cells from cocultures of *M. capsulatus* and *Chlorella sorokiniana* (Badr *et al.*, 2020). *M. capsulatus* had a consistently greater error in both dilute and concentrated samples, due to methanotroph cells sticking to microalgae cells that could not be separated properly in the flow cytometer. This yielded errors as large as 88.3% for *M. capsulatus* and 18.0% for *C. sorokiniana*, at known concentrations of 0.0367 and 0.0601 g_{DW} L^{-1} respectively. The same effect was not seen for cocultures of *Synechococcus* sp. PC7002 with *M. alcaliphilum (Badr et al., 2020)*, where cell sizes are within the same order of magnitude (Table 3.2).

The DSOF method tends to underestimate methanotroph concentrations and overestimate microalgae concentrations. This is a consequence of the DSOF method assumption that no significant amount of *Methylacidiphilum* sp. RTK17.1 cells are removed from the supernatant. Even at weak centrifugation forces, however, a fraction of methanotroph cells will be removed (Appendix C.2). If the methanotroph concentration is sufficiently dilute, however, the decrease in OD_{600} due to pelleting will be undetectable. Conversely, as concentration increases, the same fractional removal represents a greater number of cells, which thus becomes detectable by spectrophotometry. At the same time, as the method considers total coculture OD_{600} to be additive, a systematic negative error for the methanotroph concentration leads to a systematic positive error in the microalga concentration. This can be seen in Figure 3.3a, where absolute error tends to be more negative with increasing *Methylacidiphilum* concentration, while in Figure 3.3b error tends become more positive.

In order to further understand the effect of *Galdieria* sp. RTK37.1 concentration in the DSOF method, I also analysed artificial suspensions with variable microalgae concentration and constant *Methylacidiphilum* sp. RTK17.1 concentration. In Figure 3.4 I show the results of the derived concentration values as a function

of microalga [*Galdieria*]$_D$ and methanotroph ([*Methylacidiphilum*]$_D$) concentrations, for the samples where *Methylacidiphilum* OD_{600} was kept constant along each group. To visualise all the data on the same graph, I normalized the known *Galdieria* concentrations by dividing them by the corresponding *Methylacidiphilum* known concentrations. In Figure 3.4a the slopes of the derived concentration regressions should be equal to the slope of known concentration lines (as $y = mx$, with m = [*Methylacidiphilum*]$_K$). A two-tailed paired t-test revealed no significant difference (p-value > 0.05) between both set of slopes. Likewise, in Figure 3.4b the intercepts of derived concentration regressions and the known concentration lines show no significance difference (p-value > 0.05). The effect of increasing *Methylacidiphilum* concentration on the DSOF method can be clearly appreciated in Figure 3.4b, where the derived methanotroph concentrations at [*Methylacidiphilum*]$_K$ = 1.54 AU are ± 0.1 A.U. from the known values. This can also be appreciated with the relative and absolute errors in Figure 3.5. This suggests that further improvements to the DSOF method, and its extension to more concentrated suspensions, could be possible by adding a correction factor that depends on methanotroph concentration, to counteract the systematic effect of bacterial centrifugation. Figure 3.5a shows that *Galdieria* has greater error at more dilute concentrations, this is again a result of methanotroph removal from the supernatant, a small systematic effect, as seen by the absolute errors, represents a greater relative error as concentration decreases. However, most *Galdieria* relative errors are below 20%, and mostly below 10% for [*Galdieria*]$_K$ ≥ 0.50. For *Methylacidiphilum* most errors are lesser than 10%, with absolute errors mostly bound between -0.2 and 0.05 A.U. Increasing concentrations also affect errors for cell counting through flow cytometry, (Badr *et al.*, 2020) found measurements started deviating at concentrations ≥ 0.015 g_{DW} L^{-1} for *M. alcaliphilum*, ≥ 0.08, for *Synechococcus* sp. PC7002, ≥ 0.04 g_{DW} L^{-1} for *M. capsulatus*, and ≥ 0.06 *C. sorokiniana.* In contrast, in the DSOF method this happens at a concentration equivalent to 0.308 g_{DW} L^{-1} (OD_{600} =1.00 A.U.) for *Galdieria,* and 0.222 g_{DW} L^{-1} (OD_{600} = 0.5 A.U.) for *Methylacidiphilum*.

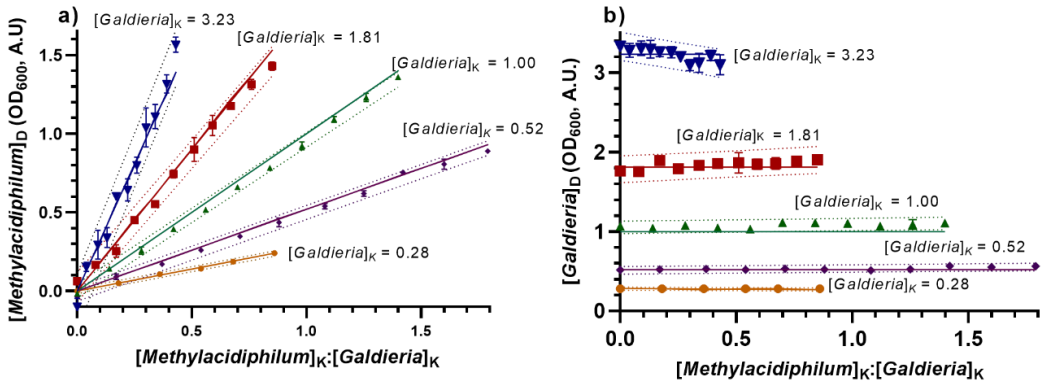


Figure 3.2. Derived concentrations of *Methylacidiphilum* sp. RTK 17.1 and *Galdieria* sp. RTK 37.1 in artificial suspensions using the DSOF method for the constant *Galdieria* concentration groups. a) Derived *Methylacidiphilum* sp. RTK17.1 ([*Methylacidiphilum*]$_D$) concentrations as a function of the known *Methylacidiphilum*:*Galdieria* concentration ratios in the artificial suspensions. b) Derived *Galdieria* sp. RTK37.1 ([*Galdieria*]$_D$) concentrations as a function of the known *Methylacidiphilum*:*Galdieria* concentration ratios in the artificial suspensions. Data points represent the derived concentrations as determined by the DSOF method; error bars represent one standard deviation (n = 3). Dashed lines represent the limit of the 95% confidence prediction bands of the derived concentrations. Solid lines represent the linear regression ($y = mx + b$) where the known *Galdieria* concentration ([*Galdieria*]$_K$) is the a) the slope $y = mx$, and b) the y-intercept $y = b$ respectively. Artificial suspensions were made by dilution of the respective stocks. For each colour group *Methylacidiphilum* sp. RTK17.1 concentrations were varied and *Galdieria* sp. RTK37.1 kept constant at the concentration shown in graphs.

Comparing the performance of the DSOF method to other methods used to quantify coculture dynamics is complicated by a lack of reported error values presented in the literature. For example, the E-C protocol (Badr *et al.*, 2020), which was analogously developed for methanotroph-phototroph cocultures, provides no data on the relative or absolute errors of the method. However, it accurately measured individual biomass concentrations in cocultures of *Methylomicrobium alcaliphilum* 20ZR (up to 0.5 g_{DW} L^{-1}) with *Synechococcus* sp. PC7002 (up to 1.0 g_{DW} L^{-1}); and in cocultures of *Methylococcus capsulatus* (up to 0.6 g_{DW} L^{-1}) with *Chlorella sorokiniana* (up to 1.5 g_{DW} L^{-1}). A good agreement was found between E-C method and flow cytometry for calculating biomass concentrations, linear relationships between the E-C method and cell counting approach had R^2 values ranging 0.90-0.98. The agreement between both approaches however deteriorated in concentrated cocultures, and the E-C method was found to be significantly better than flow cytometry at predicting total optical density for the analysed cocultures. Unlike the DSOF method, the E-C protocol does not appear to be sensitive to microorganism concentrations, at least within the reported concentrations, however it would not be a suitable method to measure relative concentrations in cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK 37.1. The E-C method is based on mass balances and the growth stoichiometry of the pair in coculture, it assumes that CO_2 is only produced by the methanotroph and only consumed by the microalgae. However, both *Methylacidiphilum* spp. and *Galdieria* sp. RTK37.1 assimilate CO_2 autotrophically via the Calvin-Benson-Bassham cycle (Khadem *et al.*, 2011), which adds an extra CO_2 sink not accounted for by the E-C method. Additionally, *Methylacidiphilum* spp. metabolic flexibility suggests the possibility of it using metabolites produced by other species (e.g. methanol and formic acid) which would modify the yields of biomass on CH_4 (Carere *et al.*, 2017; Carere *et al.*, 2021; Carere *et al.*, 2019; Pol *et al.*, 2007). This could also be the case for *Galdieria* spp., as it can grow on a variety of reduced carbon substrates (Gross & Schnarrenberger, 1995; Vítová *et al.*, 2016).

In Table 3.3 I compare several methods for determining biomass concentrations in photoautotroph-methanotroph cocultures. The DSOF method does not depend on gas concentrations, requires a small amount of liquid sample (2-3 mL), and can give results within a few minutes.

3.3.2 DSOF in live batch cocultures

The DSOF method was able to effectively quantify *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 concentrations in artificial suspensions with satisfactory accuracy and precision. However, the measured suspensions were non-growing and prepared using cells from the same growth phase (e.g. exponential), which ignores potential growth-phase dependent effects on the method. Carere *et al.* (2019) previously reported glycogen accumulation in *Methylacidiphilum* sp. RTK17.1 in response to nitrogen limitations, which might modify cell size/weight (Khadem, van Teeseling, *et al.*, 2012). Additionally, bioflocculation tendencies vary with bacterial growth phase (Powell & Hill, 2013), as there are changes to cell-surface chemistry (Powell & Hill, 2014). EPS synthesis in microalgae also vary with growth stage, with a large amount being synthesised during stationary phase (Ramanan *et al.*, 2016). Therefore, to test the DSOF method on actively growing cocultures, I monitored cell growth in batch cocultures in 1 L Pressure Bottles where: *Methylacidiphilum* growth was supressed (no CH_4), *or Galdieria* growth was supressed (no light), or neither of the microorganisms growth was supressed (Figure 3.6).

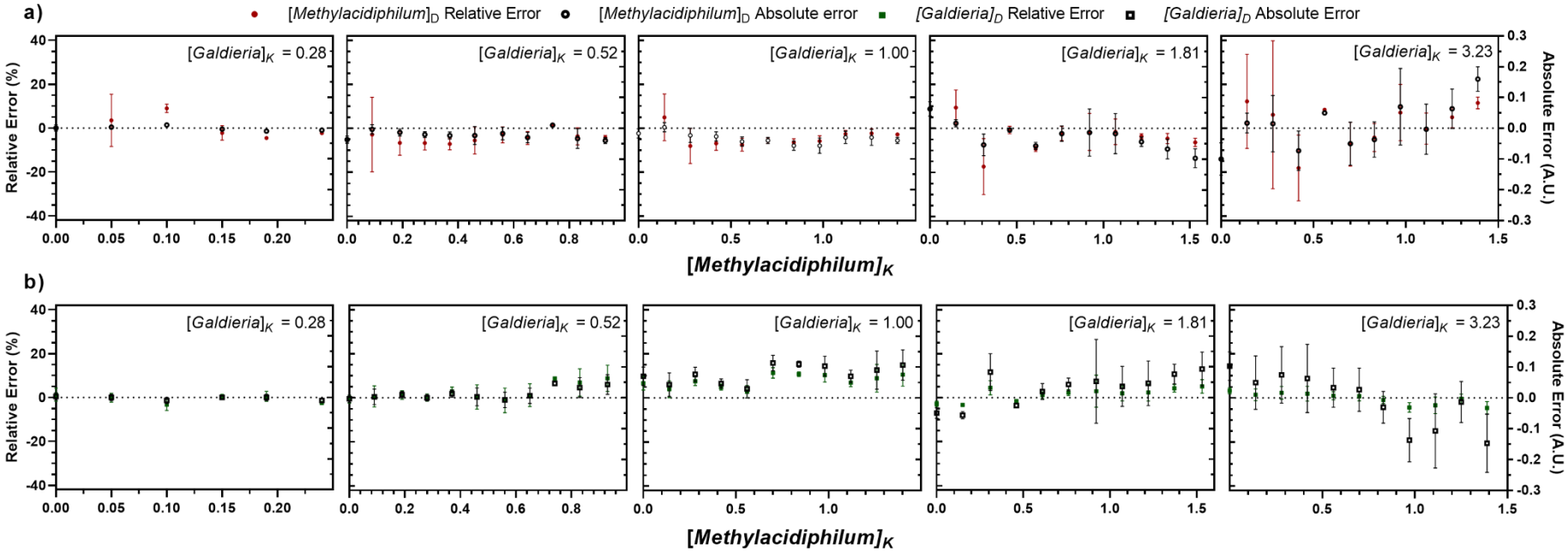


Figure 3.3. Relative and absolute errors of the derived concentrations of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK 37.1 in artificial suspensions using the DSOF method for the constant *Galdieria* concentration groups. a) Derived *Methylacidiphilum* sp. RTK17.1 relative and absolute errors as a function of the known *Methylacidiphilum* ([*Methylacidiphilum*]$_K$) concentrations in the artificial suspensions. b) Derived *Galdieria* sp. RTK37.1 relative and absolute errors as a function of the known *Methylacidiphilum* ([*Methylacidiphilum*]$_K$) concentrations in the artificial suspensions. Data points represent the relative or absolute error of the derived concentrations when compared to the known concentrations; error bars represent one standard deviation (n = 3). For each graph *Methylacidiphilum* sp. RTK17.1 concentrations were varied and *Galdieria* sp. RTK37.1 kept constant at the concentration shown. All concentrations are optical densities, in absorbance units, measured at 600 nm.

Table 3.2. Sizes of photoautotrophs and methanotrophs in commonly studied cocultures.

Photoautotroph	Size	Reference	Methanotroph	Size	Reference
Synechococcus spp.	Diameter 0.6 - 2.1 µm	(Bertilsson, Berglund, Karl, & Chisholm, 2003)	*Methylomicrobium alcaliphilum*	Rods: 1.2-1.3 µm x 2.0-3.0 µm	(Kalyuzhnaya *et al.*, 2008)
Chlorella Sorokiniana	Diameter 2 – 5 µm	(Azaman, Nagao, Yusoff, Tan, & Yeap, 2017)	*Methylococcus capsulatus*	0.7-0.9 µm wide x 0.8-1.0 µm long	(Awala *et al.*, 2020)
Galdieria spp.	Oval shape 3.8 µm x 5.0 µm	(Stadnichuk *et al.*, 1998)	*Methylacidiphilum* spp.	0.8-1.0 x 0.45-0.65	(Islam *et al.*, 2008)

As expected, derived *Methylacidiphilum* and *Galdieria* concentrations remained stable in cocultures that received 'no CH_4' and 'no light' supplementation respectively. In the 'no CH_4' experiments, measured *Methylacidiphilum* sp. RTK17.1 concentrations increased by a modest 21%, with an initial OD_{600} of 0.46 (± 0.02 A.U.), and a maximum measured concentration of 0.56 (± 0.04 A.U.) at the end of culture (Figure 3.6a). In contrast, *Galdieria* sp. RTK37.1 increased by 577% (µ = 0.017), starting at OD_{600} 0.48 (± 0.04 A.U), exhibited a 3 day lag phase, before growing to a final concentration of 2.77 (± 0.12 A.U.). For a similar range of methanotroph and microalgae concentrations in the artificial coculture experiments (Figure 3.5), the absolute errors were ± 0.10 A.U. This means that the small change in methanotroph concentration is within the method error, and no growth was detected. This is consistent with the results reported in Chapter 2; *Methylacidiphilum* RTK 17.1 does not grow in coculture with *Galdieria sp. RTK37.1* when CH_4 is depleted. Conversely, growth of *Galdieria* sp. RTK37.1 was effectively suppressed in the 'no-light' cocultures (covered in foil). The measured *Galdieria* concentrations were stable, starting at OD_{600} 0.47 (± 0.06 A.U.) and finishing at 0.46 (± 0.06 A.U.). *Methylacidiphilum* sp. RTK17.1 concentrations increased by 331% (µ = 0.0055 h^{-1}), starting at OD_{600} 0.46 (± 0.06 A.U.) and growing steadily to 1.56 (± 0.04 A.U.). The derived microalgae OD_{600} increase is within ± 0.05 A.U. (the absolute error for suspensions with a similar range of methanotroph and microalgae concentrations, Figure 3.3). This means that as expected, no microalgae growth was detected using the DSOF Method.

The DSOF method was able to effectively measure *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in the actively growing cocultures. In the cocultures where neither microorganism's growth was supressed (Figure 3.6c), *Galdieria* exhibited a lag phase of 2 days before growing from 1.01 A.U. to 2.04 (± 0.06 A.U., µ = 0.015 h^{-1}). The observed lag period is consistent with the two to three day lag phase previously reported for *Galdieria* spp. (Oesterhelt *et al.*, 2007; Sinetova *et al.*, 2006; Zhu *et al.*, 2022). *Galdieria* growth rates following the lag phase were non significantly different (*p*-value > 0.05) with the rates observed within the methanotroph-supressed 'no CH_4' coculture (Figure 3.6a). *Methylacidiphilum* concentration increased steadily from 0.31 to 1.46 A.U. (µ = 0.014), which was 2.55-fold faster than in the microalgae-supressed coculture. This can be explained by the previously observed beneficial effect on methane consumption that actively growing photoautotrophic *Galdieria* has on *Methylacidiphilum* (Section 2.3.1). When compared to our results in Chapter 2, *Galdieria* growth rate was not significantly different (*p*-value > 0.05) than in the axenic true batch growth. On the other hand, *Methylacidiphilum* exhibited a 12 % faster growth rate (*p*-value < 0.05) than in the previous axenic true batch culture (Section 2.3.1 , Figure 2.1). This makes sense, as in the DSOF coculture experiments O_2, CO_2, and CH_4 were replenished daily

to prevent nutrient limitations, while in the axenic case, true batch conditions, in concert with the stoichiometric O_2 requirement for CH_4 oxidation, led to O_2 rapidly becoming limiting. When I compare the DSOF measured methanotroph growth in this experiment, with the estimated growth in our previous work, I get similar results for the first three days of coculture (when both cocultures have available CH_4); an increase of *Methylacidiphilum* concentrations of 0.64 and 0.55 A.U. respectively, methanotroph growth rates are non-significantly different (*p*-value >0.05) between cultures. Collectively, I consider the results from these coculture experiments as evidence that the DSOF method can be used to effectively measure the relative concentrations of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in live cocultures.

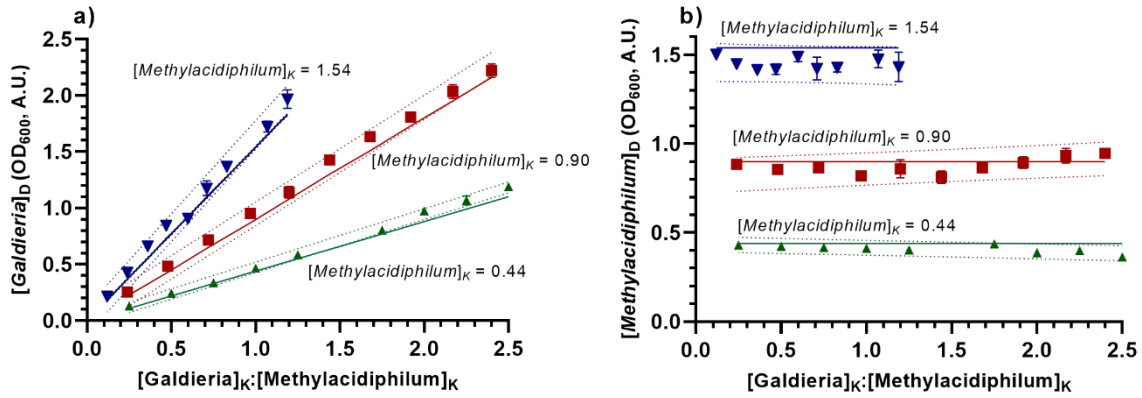


Figure 3.4. Derived concentrations of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK 37.1 in artificial suspensions using the DSOF method for the constant *Methylacidiphilum* concentration groups. a) Derived *Galdieria* sp. RTK37.1 ($[Galdieria]_D$) concentrations as a function of the known *Galdieria*:*Methylacidiphilum* concentration ratios in the artificial suspensions. b) Derived *Methylacidiphilum* sp. RTK17.1 ($[Methylacidiphilum]_D$) concentrations as a function of the known *Galdieria*:*Methylacidiphilum* concentration ratios in the artificial suspensions. Data points represent the derived concentrations as determined by the DSOF method; error bars represent one standard deviation (n = 3). Dashed lines represent the limit of the 95% confidence prediction bands of the derived concentrations. Solid lines represent the linear regression (y = mx + b) where the known *Methylacidiphilum* concentration ($[Methylacidiphilum]_K$) is a) the slope $y = mx$, and b) the y-intercept $y = b$ respectively. Artificial cocultures were made by dilution of the respective stocks. For each colour group *Galdieria* sp. RTK37.1 concentrations were varied and *Methylacidiphilum* sp. RTK17.1 kept constant at the concentration shown in graphs.

Nevertheless, while the DSOF method is quick, easy, and reliable, it still has limitations. Firstly, the method has not been validated under nitrogen limiting conditions. Both *Methylacidiphilum* spp. and *Galdieria* spp. have been found to stop cell division and accumulate glycogen under nitrogen starvation (Graziani *et al.*, 2013; Sydney *et al.*, 2019). This could increase the size and/or density of the methanotrophic cells, thus increasing their settling velocity and in consequence the number of cells separated from the supernatant. Additionally, nitrogen starved *Galdieria* cells tend to lose pigments (Salbitani & Carfagna, 2020), especially phycobiliproteins like phycocyanin, which could potentially interfere with fluorescence measurements. Both effects are likely to considerably increase measurement error, so caution is advised under nitrogen limiting conditions. As an increase in microalgae cell size would also be expected, it could be possible to decrease centrifugation time and/or force to counteract such errors. Further validation testing with cells grown under nitrogen limiting conditions is required. Furthermore, the DSOF method was only tested to total $OD_{600} \leq 5.0$ A.U., and error tended to increase with concentration. It is reasonable to expect that errors would be further aggravated with more concentrated suspensions. Adding a concentration dependant correction factor could

alleviate the problem, so could diluting the sample before centrifugation, but further research would be needed to confirm it.

Finally, because the DSOF method is based on different sedimentation speeds and pigment fluorescence measurements, it is reasonable to presume it would work with other methanotroph-photoautotroph cocultures (provided they necessarily exhibit considerable size differences). Table 3.2 shows the sizes of photoautotrophs and methanotrophs in commonly studied coculture pairs. Due to the marginal size difference between *Synechococcus* spp. and *M*. *alcaliphilum* it seems unlikely that the DSOF method would work for this pair. We would assume this to be the case for most cyanobacteria-methanotroph cocultures, due to the usually smaller sizes of unicellular prokaryotic cyanobacteria in comparison with eukaryotic microalgae. The method would likely be appropriate for C. *sorokiniana* and *M*. *capsulatus* cocultures, however it is likely parameters such as centrifugation force and/or time need would need to be optimised to maximise the efficacy of the DSOF for each coculture.

Table 3.3. Summary of the advantages and disadvantages of the various methods used to quantify biomass concentrations of individual microorganisms in photoautotroph-methanotroph cocultures.

Method	**Advantages**	**Disadvantages**
Cell counting (Flow cytometry)	High throughput.	Requires dedicated expensive equipment.
Cell counting (hemocytometry)	Inexpensive.	-Labour intensive. -Error widely varies with skill of user.
qPCR	-High precision and accuracy over multiple orders of magnitude.	-Costly reagents, time consuming, non-'realtime' processing.
EC-Method	-Error does not increase at higher biomass concentrations. -Calculates gas production and consumption rates as well as biomass concentrations. -Requires only commonly used lab equipment.	-Requires continuous monitoring of gas and liquid phase. -Requires/Assumes constant stoichiometric coefficients. -Sensitive to pressure/flow changes in coculture.
DSOF Method	-Requires small amount of sample. -Does not require gas concentrations. -Quick. -Requires only commonly used lab equipment. -Realtime processing.	-Requires size difference between phototroph and methanotroph. -Measurement error increases at high concentrations.

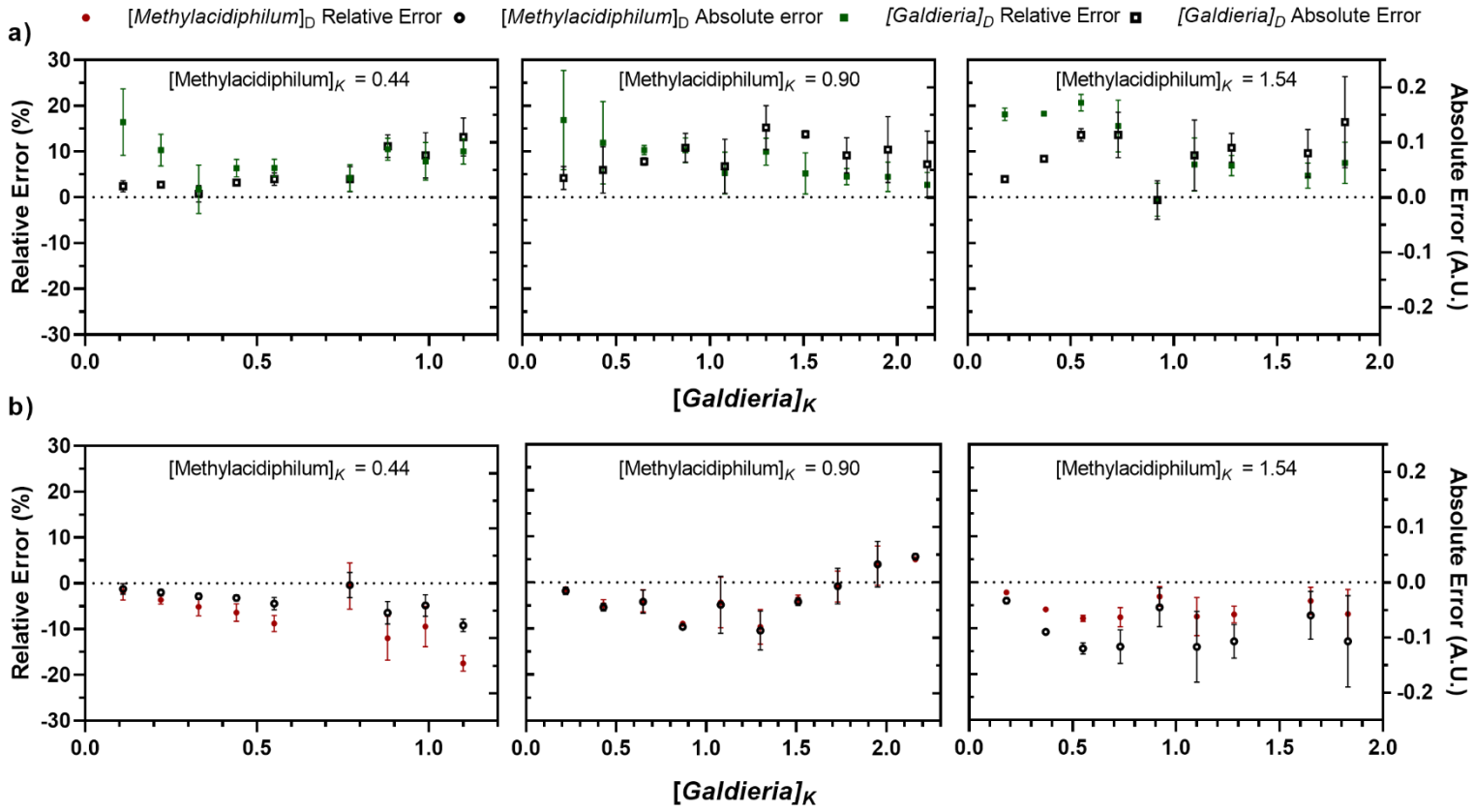


Figure 3.5. Relative and absolute errors of the derived concentrations of *Methylacidiphilum* sp. RTK 17.1 and *Galdieria* sp. RTK 37.1 in artificial suspensions using the DSOF method. a) Derived *Galdieria* sp. RTK 37.1 relative and absolute errors as a function of the known *Galdieria* ([*Galdieria*]K) concentrations in the artificial suspensions. b) Derived *Methylacidiphilum* sp. RTK 17.1 relative and absolute errors as a function of the known *Galdieria* ([*Galdieria*]K) concentrations in the artificial suspensions. Data points represent the relative or absolute error of the derived concentrations when compared to the known concentrations; error bars represent one standard deviation with n = 3. For each box *Galdieria* sp. RTK 37.1 concentrations were varied and *Methylacidiphilum* sp. RTK 17.1 kept constant at the concentration shown. The subscript “D” stands for derived value, while “K” for known value. All concentrations are optical densities, in absorbance units, measured at 600 nm.

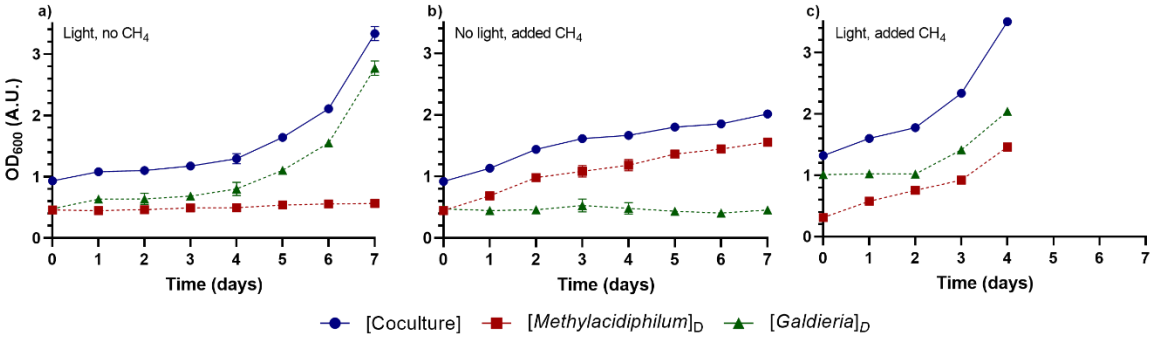


Figure 3.6. Growth of cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1. a) Coculture with no added CH_4 to suppress *Methylacidiphilum* growth. b) Coculture with added CH_4 but covered in foil to suppress *Galdieria* growth. c) Coculture with added CH_4 and no foil to allow growth of both microorganisms. Methanotroph and microalgae abundance in cocultures were calculated using the DSOF method. Cocultures were grown in V4 medium at 45 °C, 150 rpm, and with 40 µmol $m^{-2}s^{-1}$ warm white LED illumination in 1 L gastight bottles on a shaking incubator. Each day headspace gas concentrations were replenished to avoid nutrient limitations. Cultures were grown in triplicate; error bars represent one standard deviation (n = 3).

3.4 Conclusions

O_2 plays a fundamental role on *Methylacidiphilum-Galdieria* interactions, but to study these dynamics further, an accurate method to rapidly quantify the relative abundance of each microorganism was required. Consequently, a method based on differential sedimentation, optical density and autofluorescence was developed and validated in a series of artificial (non-growing) and actively growing coculture experiments. Findings show the DSOF method can predict *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 abundance with less than 0.1 A.U. absolute error when $[Galdieria]_K \leq 2.0$ A.U., and $[Methylacidiphilum]_K \leq 1.5$ A.U. These errors increase to ± 0. 2 A.U, for 2.0 A.U. < $[Galdieria]_K \leq 3.23$ A.U. Overall, the DSOF method tends to underestimate *Methylacidiphilum* concentrations, likely because the method assumes no methanotroph cells sediment during centrifugation. It is likely the DSOF method could be improved by adding a concentration dependant correction factor and, on the basis of known cell size differences, the DSOF method could likely be adapted to other methanotroph-microalgae cocultures. Collectively, the results show that the DSOF method is an easy, quick, and accurate method to measure relative concentrations of *Methylacidiphilum* sp. RTK 17.1 and *Galdieria* sp. RTK 37.1, that does not depend on gas concentrations, requires small amount of liquid sample (2-3 mL), and can give results within a few minutes. This allows for the rapid and simple measuring of the relative concentration of microalgae and methanotrophs in coculture. This novel tool enables quasi-'real time' monitoring of coculture response to different growth conditions and will help to expand understanding of photoautotroph-methanotroph cocultures and interactions.

Chapter 4. The methanotroph, *Methylacidiphilum* sp. RTK17.1, induces chlorosis in the extremophilic microalga *Galdieria* sp. RTK37.1 through oxygen limitation during photoautotrophic growth

Abstract

In Chapter 2 it was determined that *Methylacidiphilum* sp. RTK17.1 benefits from the interaction with *Galdieria* sp. RTK37.1 when in a low oxygen (O_2) environment. Under these conditions increased growth and methane oxidation rates in batch cocultures was observed. However, results also suggest O_2 plays a critical role in *Methylacidiphilum*-*Galdieria* interactions and could rapidly become a limiting factor depending on the relative concentration of each microorganism. In this study, the influence of *Methylacidiphilum*:*Galdieria* mass ratios on coculture performance, and the role of O_2 concentration in *Methylacidiphilum*-*Galdieria* interactions, was investigated. For this, several cocultures were conducted with variable initial *Methylacidiphilum*:*Galdieria* mass ratios in the presence and absence of methane (CH_4) headspace supplementation. For cocultures with dilute initial mass ratio (< 0.18 g_{DW} L^{-1}: g_{DW} L^{-1}), CH_4-containing cocultures fixed more net carbon compared to non CH_4-containing cocultures (17 – 28 % increase, *p*-value < 0.05); as the microalgae supplied the O_2 required for CH_4 oxidation. However, for CH_4-containing cocultures with initial *Methylacidphilum*:*Galdieria* mass ratios ≥ 0.23, there was a drastic reduction of growth rates (66 –100 %, *p*-value <0.001) and net carbon fixation (44 – 62 %, *p*-value < 0.001), also coproporphyrin was excreted to the media, and *Galdieria* sp. RTK37.1 exhibited chlorosis. Coproporphyrin excretion and chlorosis were preventable if O_2 was regularly replenished, and the occurrence was reversible if O_2 was permitted to accumulate. These results indicate that photoautotrophically grown *Galdieria* sp. RTK37.1 requires a minimal threshold O_2 concentration to enable adequate photo-system pigment biosynthesis. Furthermore, *Methylacidiphilum* sp. RTK17.1 exhibits a high affinity for O_2 which can induce O_2 limitation in the microalga. This is the first reported case of *Galdieria* spp. excreting coproporphyrin into the medium during photoautotrophic growth, and the first case of it being directly caused by coculture with a methanotroph. The synergistic interaction between both microorganisms becomes a deleterious interaction if the concentration of *Methylacidiphilum* sp. RTK17.1 is sufficiently high compared to *Galdieria* sp. RTK37.1.

4.1.1 Introduction

Methane (CH_4) is the second most important greenhouse gas after carbon dioxide (CO_2) (Cantera, Munoz, *et al.*, 2018). While CO_2 abatement has been the primary focus of global warming control strategies, CH_4 emission control has been proposed as an important alternative strategy (Hamer, 2010). In this sense, methanotroph-photoautotroph cocultures have attracted attention in recent years as a means to recover energy and capture carbon from both CH_4 and CO_2 (Badr, Hilliard, Roberts, He, & Wang, 2019; van der Ha *et al.*, 2011). Methanotroph-photoautotroph cocultures have also been studied for their potential to produce valuable products including biodiesel (Li *et al.*, 2022), bioplastics (van der Ha *et al.*, 2012), and single cell proteins (Rasouli *et al.*, 2018), as well as for the recovery of nutrients from industrial wastewater (Rasouli *et al.*, 2018; Roberts, Hilliard, He, & Wang, 2020; van der Ha *et al.*, 2011). Extremophilic microorganisms, including methanotrophs (e.g. *Methylacidiphilum* spp.) (Dunfield *et al.*, 2007) and microalgae (e.g. *Galdieria*

spp.) (Hirooka & Miyagishima, 2016), thrive in geothermal environments which approximate the physicochemical characteristics of many industrial processes (e.g. hot temperatures, due to the bubbling of hot flue gases, high CO_2, and acidic pH) (Donachie *et al.*, 2002; Op den Camp *et al.*, 2018). Consequently, the physiological characteristics of these extremophiles are speculated to be advantageous to cocultures at industrial scales.

One of the key interactions between methanotrophs and photoautotrophs (microalgae) in cocultures involves oxygen (O_2) production/consumption. Wherein, the photoautotroph produces O_2 as part of the water splitting reaction of oxygenic photosynthesis, the methanotroph then uses said O_2 to oxidise CH_4 for growth and energy, and in turn produces CO_2 which the photoautotroph can fix (Badr *et al.*, 2020). Methanotrophs can therefore benefit from coculture with photoautotrophs when under O_2 limitation. Several studies with different methanotroph-photoautotroph pairs have even achieved sustained methane oxidation without O_2 or air supplementation, including cocultures of the microalga *Scenedesmus* sp. with the methanotroph *Methylocystis parvus (van der Ha et al., 2012),* the cyanobacterium *Synechococcus* PCC 7002 with the methanotroph *Methylomicrobium alcaliphilum* 20z (Hill *et al.*, 2017), the microalga *Chlorella sorokiniana* with the methanotroph *Methylococcus capsulatus* (Rasouli *et al.*, 2018), and the methanotroph *Methylacidiphilum* sp. RTK17.1 with the microalga *Galdieria* sp. RTK37.1 (Section 2.3.1). An interesting O_2 interaction was described by Li *et al.* (2022), who found that culturing the mixotrophic microalga *Scenedesmus obliquus* with *Methylocystis bryophila* diminished chlorophyll concentration but increased cell density of the microalga. Presence of the bacterium also prompted downregulation of several genes associated with the Calvin Benson Bassham cycle and photosynthesis in the microalga. It was reported that unspecified reduced organic carbon was released by *M*. *bryophila,* triggering a switch from autotrophic to mixotrophic growth in *S. obliquus,* which resulted in increased biomass. At low O_2 concentrations, the microalga tended to grow autotrophically and the methanotroph would convert CH_4 into CO_2, while at higher O_2 concentrations, heterotrophic algae growth would dominate, and the methanotroph would not grow, releasing organic matter instead of CO_2 (Li *et al.*, 2022).

Previous studies have shown photoautotroph:heterotroph inoculum proportion is a key factor contributing to mixed growth performance in cocultures with microalgae (Santos & Reis, 2014). This has also been observed for cocultures of methanotrophs with other heterotrophic bacteria, for example Jeong *et al.* (2014) reported that the bacterium *Sphingopyxis* sp. NM1 stimulated growth and CH_4 oxidation rates of *Methylocystis* sp. M6 when using a 1:9 M6:NM1 volumetric ratio. This effect was not observed when the ratios were reversed (9:1 M6:NM1) or if equivalent volumetric concentrations were used (1:1). It was found that *Sphingopyxis* upregulated transcriptional expression of genes involved in methane oxidation when it was more abundant than *Methylocystis*. A similar biomass concentration dependant effect on CH_4 oxidation was observed within mixed cultures of the methanotroph, *Methylocystis* sp. M6, and the methylotrophic denitrifier, *Hyphomicrobium* sp. NM3 (Jeong & Kim, 2018).

Under O_2 limiting conditions, increased photoautotroph abundance in photoautotroph:methanotroph cocultures tends to increase growth rates when coupling methanotrophs with relatively slow-growing photoautotrophs. This has been observed for cocultures of the methanotroph *Methylococcus capsulatus* with the microalga *Chlorella sorokiniana* (Badr *et al.*, 2020), and for the methanotroph *Methylomicrobium*

buryatense 5GB1 with the cyanobacterium *Arthrospira platensis* (Badr *et al.*, 2022). In both cases, faster growth was attributed to an increase in volumetric O_2 production rate due to greater photoautotroph abundance (Badr *et al.*, 2022). This O_2 dependence was confirmed for the *M*. *buryatense* coculture with *A*. *platensis*; as external O_2 supplementation had an impact on the final photoautotroph:methanotroph ratio, and hence in *M*. *buryatense* concentration (Badr *et al.*, 2022). Under conditions of excess O_2, photoautotroph abundance in coculture does not necessarily benefit the methanotroph. Badr *et al.* (2020) reported that when coculturing the methanotroph *Methylomicrobium alcaliphilum* 20ZR with the cyanobacterium *Synechococcus* sp., the proportional abundance did not impact coculture growth rate and the limiting factor was CH_4 mass transfer (Badr *et al.*, 2020). Collectively, these results suggest the relative abundance of photoautotrophs only positively impact coculture performance under conditions of O_2 limitation.

However, not all interactions between methanotrophic bacteria and phototrophs are positive (e.g. mutualism, commensalism), for instance, Ruiz-Ruiz *et al.* (2020) found that in cocultures with excess O_2, the green microalga *Scenedesmus obtusiusculus* slowed down the CH_4 degradation rate beween 27- 43 % for an alkaliphilic methanotrophic bacteria consortium. Methane degradation was dependant on the relative ratio of S. *obtusiusculus* and the methanotroph consortium. This was attributed to the microalgae alkalizing the media, as it consumed CO_2, beyond the optimum growth pH for the bacteria (Ruiz-Ruiz *et al.*, 2020). Photoautotrophs can also be negatively affected by methanotroph presence in cocultures, as coculturing with fast growers could cause the microalgae to be outcompeted, due to shading effects and resource competition (Padmaperuma *et al.*, 2018). The microalga *Chlorella sorokiniana* has been found to have much slower apparent growth rates in cocultures with *Methylococcus capsulatus* than in comparison to axenic cultures (Rasouli *et al.*, 2018). Additionally, when subjected to low O_2 concentrations, during heterotrophic or mixotrophic growth, some species of *Galdieria* have been found to excrete the tetrapyrrole coproporphyrin III, an intermediate of chlorophyll and phycobiliproteins synthesis (Sarian *et al.*, 2016; Zhu *et al.*, 2022). This causes pigment loss and the yellowing of microalgae cells (chlorosis) (Stadnichuk *et al.*, 1998). In a coculture of mixotrophic *Galdieria sulphuraria* UTEX 2919 with a bacterial community from industrial wastewater, the additional O_2 consumption attributed to the bacterial community resulted in coproporphyrin excretion, slower growth rates, and lesser biomass titre compared to *Galdieria* grown with sterilized media (Zhu *et al.*, 2022).

As outlined above, the relative abundance of cells in methanotroph-photoautotroph cocultures can substantially impact (positively and negatively) methane consumption rates. In Chapter 2, it was demonstrated via batch coculture with *Galdieria* sp. RTK37.1, that *Methylacidiphilum* sp. RTK17.1 benefits from interspecies O_2 transfer when in a low O_2 environment (Section 2.3.1). The findings revealed, an apparent minimum O_2 concentration was required to sustain CH_4 oxidation, and it could rapidly become a limiting factor for *Methylacidiphilum*. I hypothesised that increasing the proportional abundance of *Methylacidiphilum* sp. RTK17.1 could negatively impact coculture performance and speculated a '*shading effect*' could limit photosynthesis and thus constrain coculture growth. However, extremophilic photoautotroph-methanotroph coculture dynamics remains critically understudied. Thus, in this study I investigate the influence of *Methylacidiphilum*:*Galdieria* mass ratios on coculture performance and the role of O_2 concentration in *Methylacidiphilum*-*Galdieria* interactions.

4.2 Materials and Methods

4.2.1 Stock Culture Maintenance

Methylacidiphilum sp. RTK17.1 was routinely maintained in chemostat culture using a 1 L bioreactor (BioFlo 110; New Brunswick Scientific, Edison, NJ, United States) with a 600 mL working volume and 0.0069 h^{-1} dilution rate. A gas mixture (69 % CO_2, 1.0 % CH_4, 3.1 % O_2, balance N_2, all v/v) was continuously supplied at 20 mL min^{-1}. Temperature was maintained at 45 °C, agitation at 800 rpm, and pH ~2.5 (but not controlled). Under these conditions, typical steady state OD_{600} values were 1.2 A.U. and outlet gas concentrations were 72 % CO_2, 0.13 % CH_4, 1.5 % O_2, with the remaining attributed to N_2. For all experiments, *Methylacidiphilum* sp. RTK17.1 was aseptically harvested directly from the reactor and diluted with fresh V4 media (Appendix A) to the desired starting concentration (as determined spectrophotometrically at 600 nm).

For *Galdieria* sp. RTK37.1, 300 mL stocks were repeatedly grown in batch within 1 L bottles (Duran Pressure Plus) fastened with bromobutyl rubber stoppers. At the beginning of each batch growth cycle, *Galdieria* sp. RTK37.1 cultures were diluted to OD_{600} 1.0 with sterile V4 media, and the bottle headspaces were then subjected to vacuum for 3 minutes, and re-pressurized to 5 psia with an 80 % CO_2 and 20 % (v/v) N_2 gas mixture. The bottles were then cultivated on a shaking incubator (WiseCube WIS-10, Wisd Laboratory Instruments, Germany) equipped with warm white LED strips, at 45 °C, 150 rpm, and light intensity of 40 µmol m^{-2} s^{-1} measured at the bottles' wall with a LI-250A Light Meter (LI-COR, Nebraska, United States). Growth was stopped at OD_{600} 6.0 A.U. and the cycle started anew. For all experiments, *Galdieria* sp. RTK37.1 was aseptically harvested at a OD_{600} 4.0 A.U. and diluted with fresh V4 media to the desired starting concentration.

4.2.2 Variable Mass Ratio Coculture Experiments.

Cocultures were conducted with varying initial *Methylacidiphilum*:*Galdieria* mass ratios in order to evaluate their influence on coculture performance (e.g. growth rate, biomass productivity, O_2 production rate, CH_4 oxidation rate). For these experiments, initial *Galdieria* sp. RTK37.1 concentrations (0.215 g_{DW} L^{-1}, 0.88 A.U. OD_{600}) were consistently used with varying initial *Methylacidiphilum* sp. RTK17.1 concentrations (ranging between 0.0 to 0.52 g_{DW} L^{-1}, i.e., 0.0 to 0.44 A.U. OD_{600}). Coculture suspensions (40 mL) were prepared by aseptically diluting the appropriate stocks with sterile V4 media into 160 mL serum bottles. Sufficient coculture suspension was prepared to also measure the initial total biomass concentration gravimetrically. Each serum bottle was sealed with a butyl rubber stopper and an aluminium crimp, then, to display O_2 in the headspace, flushed for 3 minutes with an 80% CO_2 and 20 % N_2 gas mixture (v/v) and pressurized to 10 psia. For each of the varying mass ratios 6 serum bottles were prepared, into which half received 20 mL CH_4 to allow growth of *Methylacidiphilum* sp. RTK17.1. N_2 (20 mL) was injected into the remaining serum bottles to serve as a non-methanotroph growing control. These preparations resulted in initial headspace concentrations of approximately 0.5 % O_2, 13.0 % CH_4, 70 % CO_2, balance N_2 (all v/v) for the CH_4 containing cocultures, and 0.5 % O_2, 70 % CO_2, balance N_2 (all v/v) for the non-CH_4 containing cocultures.

The serum bottles were then cultivated for 6 days on a shaking incubator (WiseCube WIS-10, Wisd Laboratory Instruments, Germany) at 45 °C, 150 rpm, and light intensity of 20 µmol $m^{-2}s^{-1}$. Each day, 2 mL liquid samples were collected from each bottle and analysed for total OD_{600}, pigment fluorescence, and absorption spectra measurements (discussed below in section 4.2.5). At the end of the experiment, final gauge pressure was measured, liquid and gas samples were collected, and analysed for headspace gas concentration, and final biomass concentration (Section 4.2.5).

4.2.3 Chlorosis Coculture Recovery Experiments

To investigate the impact of O_2 limitation on coproporphyrin formation, pigment loss (e.g. phycocyanin, chlorophyll) and the reversibility of chlorosis within *Galdieria* sp. RTK37.1, a series of batch cocultures were prepared with varying initial gas concentrations. For all cocultures, 250 mL of sterile V4 media were added to 1 L Duran Pressure Plus Bottles sealed with bromobutyl rubber stoppers and inoculated to the desired initial concentrations with the appropriate stocks. All bottles were cultivated in triplicate on a shaking incubator as described previously in Section 4.2.2 .

To eliminate the possibility that *Methylacidiphilum* growth by itself was sufficient to induce coproporphyrin formation and pigment loss in *Galdieria*, a series of cocultures were prepared where CH_4, CO_2 and O_2 were replenished daily. For these 'replenishment' cocultures, *Methylacidiphilum* sp. RTK17.1 was inoculated to a starting OD_{600} of 0.3 A.U. and *Galdieria* sp. RTK37.1 to an OD_{600} of 1.0 A.U. The bottles were sealed and injected with 100 mL of CO_2 and 60 mL of CH_4. To replenish gas concentrations to their initial levels, each day after sampling, all bottles were opened within a Laminar Flow cabinet, left to equalize for 30 minutes, and then re-gassed, as previously described, to the initial concentrations (Table 4.1). Growth was stopped when total OD_{600} surpassed 3.5 A.U. (Day 4).

To evaluate whether *Galdieria* sp. RTK37.1 could recover from chlorosis upon the cessation of *Methylacidiphilum* growth, a series of cocultures were prepared where first CH_4 was added and then removed once pigment loss and coproporphyrin formation was observed. *Galdieria* sp. RTK37.1 was inoculated to a starting OD_{600} of 0.35 A.U. and *Methylacidiphilum* sp. RTK17.1 to an OD_{600} of 0.80 A.U. Coculture headspaces were prepared by subjecting the bottles to vacuum for 3 minutes and then re-pressurizing to 5 psia with an 80 % CO_2 and 20 % N_2 gas mixture (v/v). The bottles were subjected to vacuum and repressurized in this manner 3 times. Following this, headspace gas was equilibrated to atmospheric pressure by expanding against a syringe, and then 120 mL of CH_4 was injected. Following 4 days incubation, all bottles were opened inside a laminar flow cabinet and left to equalise for 30 minutes. 50 mL of broth was then harvested from each bottle, centrifuged at 12,000 rpm for 15 minutes, and the supernatants were pooled together and stored at -20 °C until needed for coproporphyrin analysis. 50 mL of sterile V4 media was then added to each bottle (to maintain a 250 mL volume), bottles were re-sealed and new headspace gas compositions were prepared (as described above but without the addition of CH_4, Table 4.1) before further incubation.

A final set of cocultures were prepared with no initial CH_4, to enable comparisons of *Galdieria* growth without *Methylacidiphilum* interference. For these cocultures, *Galdieria* sp. RTK37.1 was inoculated to a starting

OD_{600} of 0.40 A.U. and *Methylacidiphilum* sp. RTK17.1 to an OD_{600} of 0.80 A.U. Coculture headspaces were prepared (as above, Table 4.1) to 20 psia with an 80 % CO_2 and 20 % N_2 gas mixture (v/v).

Table 4.1. Initial CO_2, CH_4, and O_2 headspace concentrations for the batch cocultures used in the chlorosis coculture recovery experiments.

Condition	Headspace concentrations (% v/v)*		
	O_2	CH_4	CO_2
O_2, CO_2, and CH_4 replenished each day	15.06 ± 0.09	6.94 ± 0.15	10.60 ± 0.04
CH_4 added, then removed (before CH_4 removal)	1.55 ± 0.31	12.84 ± 0.80	62.57 ± 2.83
CH_4 added, then removed (after CH_4 removal)	1.38 ± 0.41	-	60.83 ± 1.04
Methylacidiphilum growth supressed	1.30 ± 0.52	-	61.40 ± 1.00

*Balance N_2.

For all conditions 2 mL liquid and 20 mL gas samples were collected daily. Headspace pressure was measured with a pressure gauge before and after gas sampling to account for gas losses. Liquid samples were analysed via the DSOF method to assess coculture dynamics, in addition to total OD_{600}. Pigment fluorescence, and absorption spectra were also measured. For all conditions, cultures were stopped when any of the replicates total OD_{600} surpassed 3.5 A.U. to avoid N-limitation.

4.2.4 Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

To confirm the identity of coproporphyrin in culture supernatants, samples were subjected to LC-MS analysis. For these measurements, a 500 µL aliquot of the aqueous centrifuged supernatant (Section 4.2.3) was diluted 1:1 in isopropanol (IPA) and filtered through a 0.22 µm PTFE syringe filter. Samples were then analysed on an Agilent UPLC-QTOF spectrometer equipped with an Agilent 1290 high speed binary pump, Agilent 1290 multi-sampler, Agilent 1290 multi-column thermostat, and Agilent 1260 diode array detector, and Dual AJS ESI source in positive ion mode. The LC method used an Agilent Zorbax Eclipse Plus 50 x 2.1 mm, 1.8 µm column, 35 °C temperature, 20 minutes run length, positive MS polarity, 10 µL injection volume, 0.3 mL min^{-1} flow rate. An acetonitrile/water gradient (with 0.1% formic acid) was used. The gradient started at 2 % acetonitrile (with 0.1 % formic acid) and 98% water (with 0.1 % formic acid) for 15 minutes, then increased to 100% acetonitrile (with 0.1 % formic acid) for 5 minutes. A 1:1 IPA in water (LCMS grade) solution was used as a blank.

LCMS data was analysed using Agilent MassHunter Qualitative analysis 10.0 software. The Base Peak Chromatogram (BPC) was generated for each sample and the relevant blank subtracted. Mass spectra were extracted for the relevant peaks and the mass spectrum background (from the blank) subtracted. An Extracted Ion Chromatogram (EIC) for the expected ions for coproporphyrin III ($[M+H]^+$ = 655.2762 and $[M+Na]^+$ = 677.2582) was generated in positive ion mode. Formulas for the ions in the peak at retention time 7.46 min were generated to see if there was a good match for coproporphyrin III.

4.2.5 Analytical Procedures

Optical density at 600 nm was routinely measured using an Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom). To differentiate the contribution of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 to the coculture OD_{600}, the DSOF method (Section 3.2.2) was used.

Variation in pigments in coculture were tracked by autofluorescence. For these measurements, 200 µL samples were transferred into black, flat bottom (chimney well) 96-well microplates (Greiner Bio-One, Austria). Fluorescence was measured at 670 nm emission (590 nm excitation) for phycocyanin (Gregor & Maršálek, 2005) and at 670 nm emission (485 nm excitation) for chlorophyll (Gregor & Maršálek, 2005) in a plate reader spectrophotometer (Varioskan Lux, Thermo Scientific, United States). For the absorption spectra, 200 µL samples were transferred into clear 96-well microplates (Greiner Bio-One, Austria), and optical density was measured between 300 to 800 nm (in 1 nm increments) using a plate reader spectrophotometer (Varioskan Lux, Thermo Scientific, United States). To quantify coproporphyrin variation, the average of the optical density values at 390 and 410 nm was subtracted from the 400 nm coproporphyrin peak. Negative values calculated in this fashion were considered to be 0.

Biomass concentrations were measured gravimetrically. For these, 15 mL aliquots were vacuum-filtered on pre-weighted 0.22 µm nylon membrane filters (MicroScience, Australia). The filters were then washed 3 times by vacuum-filtering 10 mL of deionized water. The filters were dried for 12 hours at 95 °C, weighed, and the filtered biomass weight calculated by difference.

Gas samples were analysed for CO_2, CH_4, N_2, and O_2 concentrations using a 490 micro GC equipped with a thermal conductivity detector (Agilent Technologies, United States), equipped with Molecular Sieve 5A with a heated injector (50 °C, back-flush at 5.10 s, column at 90 °C, 150 kPa), a PoraPak Q column with a heated injector (50 °C, no back-flush, column at 70 °C, 50 kPa) and a 5CB column with a heated injector (50 °C, no back-flush, column at 80 °C, 150 kPa).

All Statistical analyses were performed using Prism Graphpad 9.4.1. Unless stated otherwise, Two-factor ANOVA ($\alpha = 0.05$) tests were used, with Sidak method for multiple mean comparisons between columns (simple effects within rows) or rows (simple effects within columns). Growth rates were calculated by fitting the appropriate concentration data to the exponential growth equation model in Prism Graphpad 9.4.1 ($Y = Y_0 e^{\mu_{max} t}$, where Y is the concentration in A.U., Y_0 is the concentration at time zero in A.U., μ_{max} is the specific growth rate in h^{-1}, and t is time in h).

4.3 Results and discussion

4.3.1 Initial Batch Coculture Mass Ratio Influences Biomass Productivity.

The relative proportion of member species in photoautotroph:methanotroph cocultures is known to significantly influence bioprocess performance, however no studies have yet investigated the impact of this parameter within thermoacidophilic cocultures. Therefore, to evaluate the influence of the *Methylacidiphilum*:*Galdieria* mass ratios on coculture performance, several batch coculture experiments

were performed with varying initial biomass ratios. In the cocultures that did not receive CH_4, *Methylacidiphilum* sp. RTK17.1 was predictably unable to grow, and therefore increasing its initial concentration had minimal impact on the observed coculture growth rate, which was attributed to *Galdieria* sp. RTK37.1 growth (Figure 4.1a). Nevertheless, a small but significant decrease (9%, *p*-value < 0.005) in the overall growth rate was observed with increasing *Methylacidiphilum* sp. RTK17.1 proportional abundances, which I attribute to a shading effect. Shading by bacteria in coculture has previously been shown to negatively impact photoautotroph growth, in cocultures of the methanotroph *Methylomicrobium* with the cyanobacteria *Arthrospira platensis* (Badr *et al.*, 2022). This effect is better appreciated, in our cultures, when comparing rates of biomass productivity: a maximum productivity of 250 (± 3) mg_{DW} L^{-1} h^{-1} was obtained at an initial 0.08 g_{DW} L^{-1}: 1 g_{DW} L^{-1} *Methylacidiphilum*:*Galdieria* ratio, while the minimum productivity of 188 (± 4) mg_{DW} L^{-1} h^{-1} was obtained when *Methylacidiphilum* sp. RTK17.1 was most abundant (Figure 4.1b). In contrast, the performance of cocultures (both growth rate and biomass productivity) with added CH_4 dramatically decreased with increasing *Methylacidiphilum*:*Galdieria* mass ratios. In cocultures with mass ratios ≤ 0.18, a small continuously slowing of coculture growth rate (8 % decrease, *p*-value < 0.01) was observed. Increasing the initial *Methylacidiphilum* concentration any further, however, slowed growth significantly (*p*-value < 0.001), with no growth observed at a mass ratio of 1.33 g_{DW} L^{-1}: g_{DW} L^{-1}. This result cannot be explained solely by cell shading, as the effect was not as intense in the cocultures without CH_4. Increasing *Methylacidiphilum* concentration 7.4-fold (*Methylacidiphilum*:*Galdieria* ratio 0.18 to 1.33) slowed the overall growth rate by only 11 %; while in the CH_4 containing cocultures, increasing *Methylacidiphilum* concentration 1.3-fold (mass ratio 0.18 to 0.23) slowed the overall growth rate by 66 % (Figure 4.1).

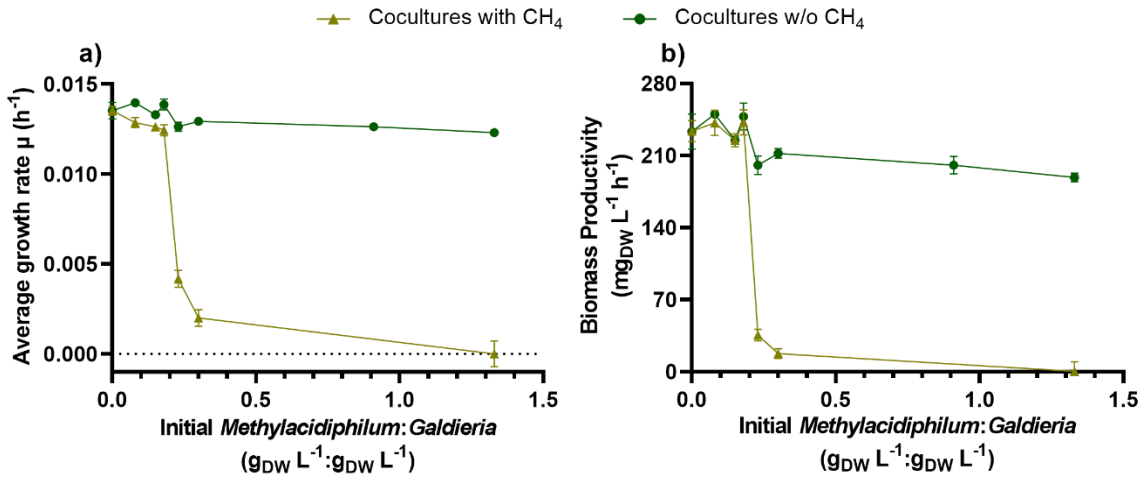


Figure 4.1. Effect of the initial *Methylacidiphilum* RTK17.1 to *Galdieria* RTK37.1 concentration ratio on coculture growth, for cultures with and without CH_4 supplementation into the headspace. The a) average growth rate was calculated over the entire culture period using the initial and final biomass concentrations. For the cocultures without CH_4 supplementation, *Methylacidiphilum* concentration was substracted to the initial and final concentrations, as it does not contribute to biomass growth. b) biomass productivity. Residual oxygen at the start of cultures ranged from 0.1 to 2.4% (v/v). Cultures grown in 160 mL serum bottles in a shaking incubator at 45 °C, 110 rpm, and 20 µmol m^{-2} s^{-1} measured at the bottle wall. Error bars represent a standard deviation (n = 3).

Biomass productivities (Figure 4.1b) indicate that, for initial mass ratios ≥ 0.23 (*Methylacidiphilum*:*Galdieria*), much less biomass was produced when CH_4 was available. Evidently

Methylacidiphilum sp. RTK17.1 growth can impede *Galdieria sp.* RTK37.1 growth. These decreased productivities contrast those reported by Badr *et al.* (2020) in batch cocultures involving different methanotroph-photoautotroph pairs (*Methylomicrobium alcaliphilum* 20ZR / *Synechococcus* sp. PCC7002; *Methylococcus capsulatus* / *Chlorella sorokiniana*) with varying biomass ratios. For *M*. *capsulatus* / *C*. *sorokiniana* cocultures, greater proportions of microalgae tended to increase coculture growth rates, as the comparatively slow growing *C*. *sorokiniana* produced limited O_2 (via photosynthesis) and hence constrained methanotroph growth. This is similar to the increased growth and CH_4 oxidation rates that I observed previously for *Methylacidiphilum* sp. RTK17.1 when grown in coculture with *Galdieria* sp. RTK37.1 (Section 2.3.1 , Figure 2.1, Figure 2.3); and hence what was expected in these differential *Methylacidiphilum*:*Galdieria* ratio experiments. For *M*. *alcaliphilum* / *Synechococcus* cocultures, using a greater cyanobacterium concentration did not impact coculture growth, as the cyanobacterium grew much faster than the methanotroph, which was thusly limited by CH_4 mass transfer rather than O_2 availability (Badr *et al.*, 2022).

In addition to decreassed biomass productivities, all cocultures grown with CH_4 at an initial *Methylacidiphilum*:*Galdieria* mass ratio ≥ 0.23 developed a yellow coloration within 4 days incubation (Figure 4.2). In microalgae N-starvation is known to trigger a similar colour change, known as 'chlorosis', which is caused by the loss of chlorophylls and phycobiliproteins (Salbitani & Carfagna, 2020). Chlorosis due to N-starvation is well documented for photoautotrophic and heterotrophic *Galdieria* spp. (Sinetova *et al.*, 2006) and the loss of pigments can be very detrimental to microalgae growing photoautotrophically, as it negatively impacts their ability to capture light and perform efficient photosynthesis (Salbitani & Carfagna, 2020). In this study excess ammonium was supplied to the cocultures (Section 2.3.1 ,Figure 2.2), therefore the chlorosis observed (and slow growth), is not attributable to N-limitation. A previous study by Li *et al.* (2022) demonstrated methanotrophs can affect pigment concentration in microalgae, wherein culturing the microalga *Scenedesmus obliquus* with *Methylocystis bryophila* diminished chlorophyll concentration but increased cell density of the microalga. Coculturing *S. obliquus* with the methanotroph bacteria induced the downregulation of genes associated with the Calvin Cycle and photosynthesis in the microalga. It was concluded that the organic carbon metabolites released by *M*. *bryophila* triggered a switch from autotrophic to mixotrophic growth in *S. obliquus*, which explained the increase in biomass. While *Galdieria sulphuraria* is known to grow mixotrophically (Liu *et al.*, 2021; Zhu *et al.*, 2022), growth rates are typically faster than for autotrophic growth (Henkanatte-Gedera *et al.*, 2017), so a switch to mixotrophic growth seems unlikely.

Net CO_2 consumption rates were ~80 % slower in the cocultures that developed yellow coloration compared to the corresponding non-CH_4 cocultures of equivalent mass ratio (p-value < 0.0001) (Figure 4.3a). While this observation is partially explained by CO_2 produced via CH_4 oxidation (Figure 4.3b), the trend also holds for net C fixation (44 – 62% slower net C fixation rate, p-value < 0.001) (Figure 4.3c). This means that in CH_4-containing cocultures with an initial mass ratio ≥ 0.23 (*Methylacidiphilum*:*Galdieria*), *Galdieria* consumed less CO_2, which was consistent with the lesser biomass productivities. CH_4 consumption in these cocultures is interesting, as increasing *Methylacidiphilum* starting concentrations coincided with increased net CH_4 oxidation rates to a maximum of 0.124 ± 0.001 $mmol_{CH4}$ L^{-1} h^{-1} at a *Methylacidiphilum*:*Galdieria* ratio of 0.15 (1.4-fold increase, p-value < 0.05). At this ratio CH_4 was completely removed, so it is possible

faster consumption rates would have been observed if additional CH_4 had been provided. However, further increasing the *Methylacidiphilum* concentration in cocultures tended to diminish the amount of CH_4 removed. A similar result was reported by Ruiz-Ruiz *et al.* (2020) in cocultures involving an alkaliphilic methanotrophic bacterial consortium (AMB) and the green microalgae *Scenedesmus obtusiusculus* (GM). Cocultures at a 3:1 AMB:GM mass ratio exhibited the fastest CH_4 degradation rate and greatest methane removal (393 ± 0.013 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}, 65.3% removal) when compared to 1:1 (271 ± 0.054 mg_{CH4} $g^{-1}_{biomass}$ d-1, 19.0% removal) and 1:3 ratios (271 ± 0.054 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}, 13.0% removal). The authors contend that in ratios with greater GM, the microalgae alkalized the media beyond the optimum growth pH for the methanotrophic bacterial consortium. In a second round of experiments the 3:1 ratio again showed a maximum in CH_4 degradation rate (589 ± 0.011 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}) when compared to a 4:1 (540 ± 0.015 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}) and 5:1 ratio (490 ± 0.023 mg_{CH4} $g^{-1}_{biomass}$ d^{-1}). No explanation for the slowdown at the greater methanotroph concentration (e.g. 5:1 ratio) was provided. AMB controls without microalgae exhibited faster degradation rates than any coculture, since excess O_2 was supplied for all cultures, it is possible that the methanotrophic consortia did not benefit from the microalgae presence.

Figure 4.2. Comparison of cocultures with an initial concentration ratio of 0.30 *Methylacidiphilum*:*Galdieria* without CH_4 (left), and with CH_4 (right). Picture taken at the end of the culture (day 6). All cocultures grown with CH_4 at an initial mass ratio ≥ 0.23 developed a similar yellow coloration within 4 days incubation. Cultures grown in 160 mL serum bottles in a shaking incubator at 45 °C, 110 rpm, and 20 µmol m^{-2} s^{-1}.

In this work, the decrease in CH_4 consumption is interpreted as a consequence of limited O_2 availability, as the net O_2 production (Figure 4.3d) was 56 - 99 % lesser (*p*-values < 0.0001) for CH_4-containing cocultures with initial *Methylacidiphilum*:*Galdieria* ratios > 0.15 (and approached 0 for ratios ≥ 0.23 g_{DW} L^{-1}: g_{DW} L^{-1}). At the highest ratios, some CH_4 oxidation was observed (0.062 ± 0.016 $mmol_{CH4}$ L^{-1} h^{-1}). *Galdieria* therefore must have initially been capable of O_2 production. However, the produced O_2 was quickly consumed by *Methylacidiphilum*, which caused O_2 limitation. As a result, OD_{600} increased only within the first 24 h of cultivation for CH_4-containing cocultures with initial ratios > 0.15 (Appendix D). The subsequent appearance of the yellow colouration (e.g 1-2 days post O_2 limitation) is consistent with photopigment damage (chlorosis) and a cessation of oxygenesis by *Galdieria*. The beneficial interaction on CH_4 oxidation that *Galdieria* sp. RTK37.1 confers to *Methylacidiphilum* sp. RTK17.1 in low O_2 environments seems to depend on their relative concentrations. In this experiment initial *Methylacidiphilum*:*Galdieria* mass ratios ≤ 0.15

allowed for greater carbon fixation, while ratios ≥ 0.23 g_{DW} L^{-1}: g_{DW} L^{-1} negatively impacted *Galdieria* and hence coculture growth, CO_2 fixation and CH_4 oxidation. The concentration of O_2 also had a defining role in gas consumption/production in the cocultures between *M*. *bryophila* and *S*. *obliquus* (Li *et al.*, 2022). At low O_2 concentrations the microalga tended to grow autotrophically and the methanotroph would convert CH_4 into CO_2, while at high O_2 concentrations, heterotrophic algae growth would dominate and the methanotroph would release organic matter instead of CO_2. (Li *et al.*, 2022).

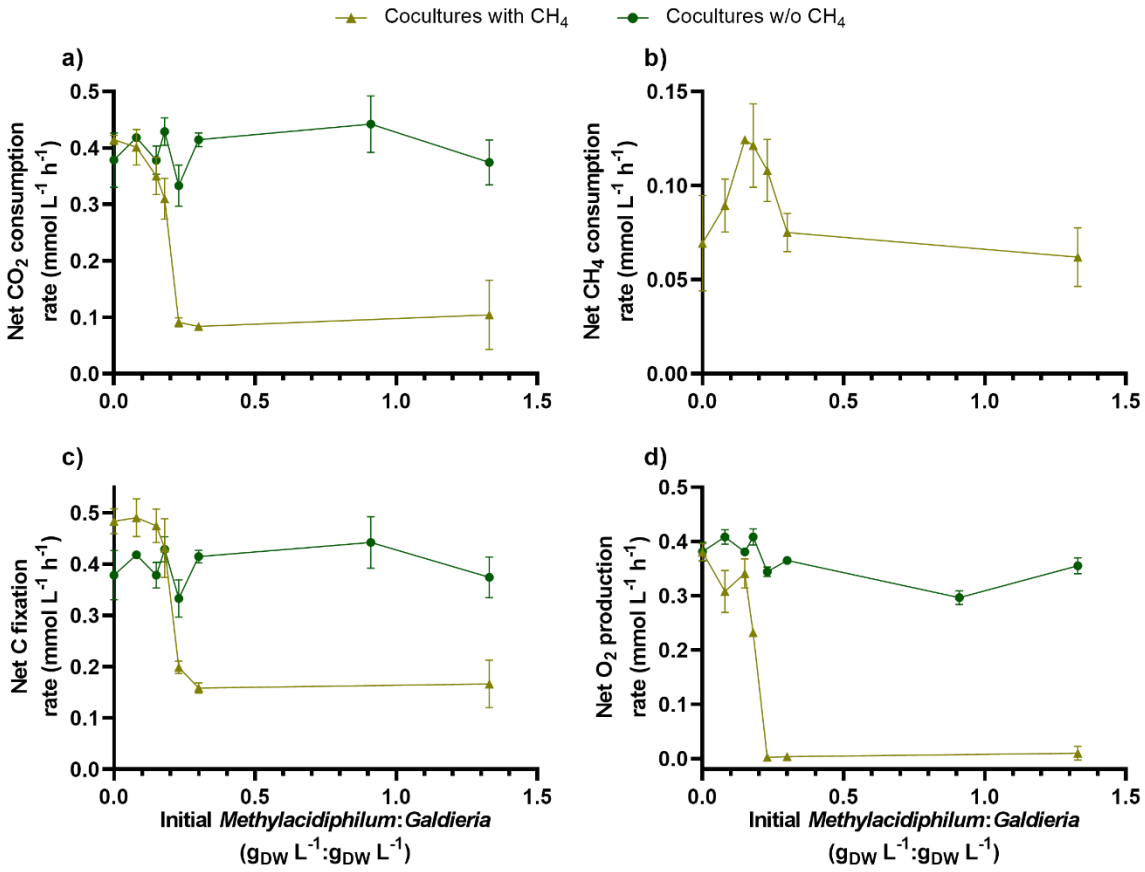


Figure 4.3. Effect of the initial *Methylacidiphilum* sp. RTK17.1 to *Galdieria* sp. RTK37.1 mass ratio on net production and consumption rates, for cocultures with and without supplemental CH_4. The a) net CO_2 consumption rate, b) net CH_4 consumption rate, c) net carbon (C) fixation rate and d) net oxygen (O_2) production rates are shown. All rates were calculated over the entire culture period (6 days) using the initial and final gas concentrations, pressures and temperatures. Cocultures were grown in 160 mL serum bottles in a shaking incubator at 45 °C, 110 rpm, and 20 µmol m^{-2} s^{-1} measured at the bottle wall. Error bars represent one standard deviation (n = 3).

Both the appearance of the yellow colouring as well as the diminished O_2 production rates are consistent with pigment degradation of *Galdieria* in coculture. In order to further investigate pigment content (e.g. chlorophyll, phycocyanin) of *Galdieria* sp. RTK37.1 cells, cocultures were assessed spectrophotometrically across 300-800 nm. Absorption maxima at 436 and 678 nm correspond to chlorophyll (Sinetova *et al.*, 2006)**,** and at 624 nm to phycocyanin (Sinetova *et al.*, 2006; Stadnichuk *et al.*, 2011). The absorption spectra for CH_4-containing cocultures with ratios ≥ 0.23 (Figure 4.4a) show that as coculture progresses, the chlorophyll, and phycocyanin content in *Galdieria* sp. RTK37.1 decreases, which signals pigment degradation. The absorption spectra for all cocultures at mass ratios < 0.23 were each consistent with healthy growing *Galdieria* (e.g. peaks associated with chlorophyll and phycocyanin continued to develope

throughout cultivation). Concurrent with pigment loss, spectrophotometirc analysis revealed the appearance of a peak at 400 nm (Figure 4.4a inset). This peak is associated with presence of coproporphyrin in solution (Cleary, Kolachina, Wolfe, & Sanchez, 2018), and LC-MS spectra of supernatant samples is consistent with coproporphyrin III presence (Appendix E). Coproporphyrin III has been reported to be excreted by heterotrophic or mixotrophic cultures of *Galdieria partita* (Stadnichuk *et al.*, 1998), *Galdieria sulphuraria* strain 074G (Sarian *et al.*, 2016), and *Galdieria Sulphuraria* UTEX 2919 *(Zhu et al., 2022)* under O_2 limitation.

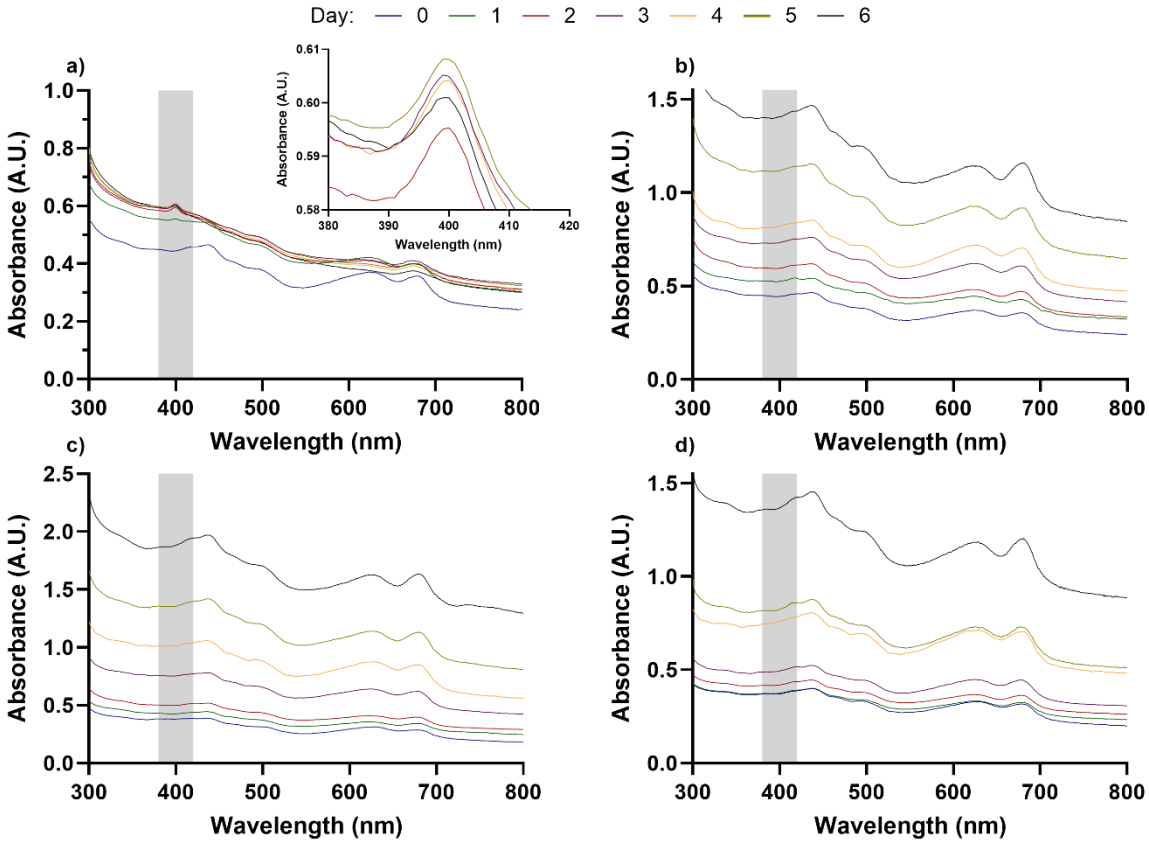


Figure 4.4. Absorption spectra for representative cocultures. a) CH_4-containing cocultures with an initial *Methylacidiphilum:Galdieria* mass ratio of 0.30, the inset displays a magnified view between 380 and 420 nm to highlight the coproporphyin peak at 400 nm. b) Non CH_4-containing cocultures with initial *Methylacidiphilum:Galdieria* mass ratio of 0.30 c). CH_4-containing cocultures with initial *Methylacidiphilum:Galdieria* mass ratio of 0.15, and d) CH_4-containing cocultures *Methylacidiphilum:Galdieria* mass ratio of 0. The grey shaded area between 380 and 420 nm was added to highlight the presence or abscense of the coproporphyin peak at 400 nm. Optical densities were measured in 1 nm increments, all absortion spectra were generated from the average optical densitiy values obtained from triplicate cocultures.

Coproporphyrin is an intermediate formed during the biosynthesis of both phycocyanin and chlorophyll (Stadnichuk *et al.*, 1998). During normal pigment biosynthesis, the precursor coproporphyrinogen III is oxidised by an oxygen oxidoreductase (coproporphyrinogen III oxidase), HemF (E.C. 1.3.3.3), into protoporphyrinogen IX (Sarian *et al.*, 2016; Skotnicová *et al.*, 2018). HemF is completely inhibited by anaerobic condition, so under oxygen limitation coproporphyrinogen III acumulates, and is eventually excreted, where the acidic media oxidizes it into coproporphyrin III (Sarian *et al.*, 2016). As a consequence, pigment synthesis stops. In *Galdieria sulphuraria*, supression of phycobiliprotein synthesis during N-

starvation induced chlorosis leading to complete phycocyanin degradation within 3-4 days (Sinetova *et al.*, 2006). While O_2 limitation seems counterintuitive for a photoautotroph, and there is no report on coproporphyrin formation in autotrophic *Galdieria* spp., coproporphyrin formation has been shown to only occur under O_2 limiting conditions in both heterotrophic and mixotrophic *Galdieria* spp. cultures (Sarian *et al.*, 2016). In these coculture experiments, O_2 limitation is likely precipitated by the combination of high relative abundance of *Methylacidiphilum* sp. RTK17.1, the relatively fast O_2 consumption rate for this strain (2.33 ± 0.19 mmol g_{DW}^{-1} h^{-1} O_2 consumption for *Methylacidiphilum* vs. 0.966 ± 0.152 mmol g_{DW}^{-1} h^{-1} O_2 production for *Galdieria* sp. RTK37.1 in chemostat culture) (Section 2.3.2), and an exceptionally strong oxygen affinity (K_s = 0.7 mM) characteristic of verrucomicrobial methanotrophs (Pol *et al.*, 2007). A similar effect was recently reported in a coculture of mixotrophic *Galdieria sulphuraria* UTEX 2919 with a bacterial community from industrial wastewater (Zhu *et al.*, 2022). In these cocultures, the increased O_2 demand due to bacterial activity resulted in coproporphyrin excretion, slower growth rates and lesser biomass titre compared to axenically grown *Galdieria* (Zhu *et al.*, 2022).

In order to more easily compare pigment content changes in the cocultures, I measured chlorophyll and phycocyanin autoflorescense. Within cocultures with *Methylacidiphilum:Galdieria* initial mass ratios ≤ 0.18, the chlorophyll (Figure 4.5a), and phycocyanin profiles (Figure 4.5b) were consistent with normal batch photoautotrophic growth: a 2-3 day lag phase, followed by continuous increase, and then a stationary or decay phase once ammonium was depleted from the medium (Oesterhelt *et al.*, 2007; Sinetova *et al.*, 2006). No coproporphyrin (Figure 4.5c) was released into the media under these conditions, as excess O_2 was available. In contrast, for cocultures with *Methylacidiphilum:Galdieria* mass ratios ≥ 0.23, the methanotroph quickly depleted available O_2, triggering its limitation in *Galdieria*. Coproporphyrin excretion into the medium was observed within 2 days for the cocultures with an initial ratio of 0.23, and within 24 hours for the cocultures with greater *Methylacidiphilum:Galdieria* mass ratios. As coproporphyrin excretion detracts from the available coproporphyrinogen III pool (Sarian *et al.*, 2016), pigment biosynthesis stopped and phycocyanin degraded. Concurrent to phycocyanin concentration decreasing, the yellow coloration started to appear between days 2-4 of culture. Chlorophyll concentration remained stable for 3-4 days, but its concentration eventually started diminishing, albeit slowly. This behaviour also occurs for N-starvation induced chlorosis in *Galdieria sulphuraria,* phycobiliproteins are degraded completely 3-4 days after nitrogen deficiency, while chlorophylls and carotenoids start decreasing after 4 days (Sinetova *et al.*, 2006). Then on the onset of chlorosis in the cocultures, pigment damage impeded photosynthesis, and as a result *Galdieria* growth stopped; with no further O_2 produced. As O_2 was no longer available, methane oxidation could not be sustained, and hence *Methylacidiphilum* growth also stopped. In low O_2 environments, therefore, *Methylacidiphilum* sp. RTK17.1 can inhibit *Galdieria* sp. RTK37.1 growth by triggering O_2 limitation, which leads to coproporphyrin formation and chlorosis in the microalga.

4.3.2 *Methylacidiphilum* sp. RTK17.1 induces O_2 limitation and subsequent chlorosis in *Galdieria* sp. RTK37.1.

To further understand the role of O_2 concentration in coproporphyrin formation and pigment loss in cocultures, a series of batch cocultures were performed in 1 L pressure bottles with different gas concentrations. For all cocultures (with and without CH_4 supplementation), an initial

Methylacidiphilum:Galdieria mass ratio of ~ 1.33 was used. In the cocultures where no CH_4 was added (e.g. *Methylacidiphilum* growth suppression), after a 2-day lag phase *Galdieria* sp. RTK37.1 growth (Figure 4.6a) was not significantly different (*p*-value > 0.05) than previously observed axenic *Galdieria* (Section 2.3.1). Pigment fluorescence (Figure 4.7a) followed the same trends as the CH_4-containing serum bottles cocultures with *Methylacidiphilum:Galdieria* ratios ≤ 0.18. This contrasts with growth in the CH_4-containing cocultures (Figure 4.6c), where no *Methylacidiphilum* sp. RTK17.1 growth was observed (OD_{600} values showed no significant change, *p*-value > 0.05) and *Galdieria* sp. RTK37.1 and OD_{600} increased sligthly from 0.81 ± to 1.00 ± 0.04 A.U. on the first day of culture (*p*-value < 0.0001) and then stagnated. Also phycocyanin (Figure 4.7c) started degrading on the first day of culture, and chlorophyll on the fourth. In summary, G*aldieria* sp. RTK37.1 growth and pigment production were similar during axenic growth, during coculture with non-growing *Methylacidiphilum* sp. RTK17.1, and during coculture with low concentrations of actively growing *Methylacidiphilum* sp. RTK17.1. Collectively, these results suggest that, aside from a modest shading effect, *Methylacidiphilum* sp. RTK17.1 does not affect the (photoautotrophic) growth of *Galdieria* sp. RTK37.1, unless their relative concentration are sufficient to induce CH_4-oxidation dependent O_2 limitation.

To eliminate the possibility that *Methylacidiphilum* growth by itself was sufficient to induce coproporphyrin formation and pigment loss in *Galdieria*, a series of cocultures were prepared where CH_4, CO_2 and O_2 were replenished daily. In these cocultures, the total final OD_{600} (Figure 4.6b) reached 3.5 A.U. within 4 days incubation. Despite *Methylacidiphilum* being much more concentrated than in the cocultures without CH_4-addition (Figure 4.6a), *Galdieria* growth in both was similar, a 2-day lag phase followed by OD_{600} doubling in another 2 days, there was no significant difference in growth rates (*p*-value > 0.05). Additionally, chlorophyll and phycocyanin (Figure 4.7b) accumulated throughout cultivation, and no coproporphyrin was observed. Thus, in the presence of excess O_2, the growth of *Methylacidiphilum* sp. RTK17.1 is not sufficient to inhibit *Galdieria*, as even at methanotroph OD_{600} > 1.0 A.U. no signs of chlorosis were observed.

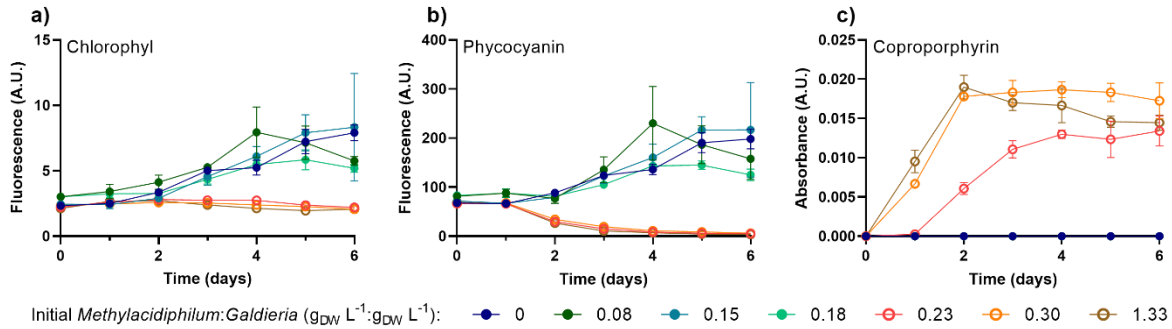


Figure 4.5. Temporal profiles for a) chlorophyll (670 nm emission, 485 nm excitation), b) phycocyanin (670 nm emission, 485 nm excitation), and c) coproporphyrin within CH_4-containing cocultures with varying initial *Methylacidiphilum:Galdieria* mass ratios. Coproporphyrin peak absorbance was measured by substracting the average of the optical densities at 390 and 410 from the 400 nm peak. Negative values calculated in this fashion were considered to be 0. Closed circles represent cocultures with initial *Methylacidiphilum:Galdieria* ratio ≤ 0.18 (non coproporphyrin producing), while open circles represent cocultures with initial mass ratios ≥ 0.23 (coproporphyrin producing). Cultures were grown in 160 mL serum bottles in a shaking incubator at 45 °C, 110 rpm, and 20 µmol m^{-2} s^{-1} measured at the bottle wall. Error bars represent one standard deviation of biological triplicates (n = 3).

4.3.3 O_2-Limiting chlorosis is reversible in *Galdieria* sp. RTK37.1.

To evaluate whether *Galdieria* sp. RTK37.1 could recover from pigment loss (e.g. chlorophyll, phycocyanin) in cocultures, a series of cocultures were prepared where first CH_4 was added and then removed following pigment loss and coproporphyrin formation (day four, Figure 4.8). Photographs (Figure 4.9) show that the yellow pigmentation appeared as phycocyanin was almost depleted on day two. Following the removal of CH_4, a period of pigment recovery (without growth, days four to seven) ensued (Figure 4.10). This period was characterised by a white coloration, as chlorophyll contents continued to decrease for two days following CH_4 removal. Concurrent to the cessation of *Methylacidiphilum* growth, coproporphyrin concentration started decreasing. The rapid disappearance of coproporphyrin cannot be properly explained by its decomposition alone, as its concentration in the differential *Methylacidiphilum*:*Galdieria* ratio experiments (Section 4.3.1 , Figure 4.5) stabilized at a similar concentration for at least four to six days. Furthermore, Sarian *et al.* (2016) showed no coproporphyrin degradation in the media after 15 days of reaching the stationary phase in an oxygen limited mixotrophic culture of *Galdieria sulphuraria*.

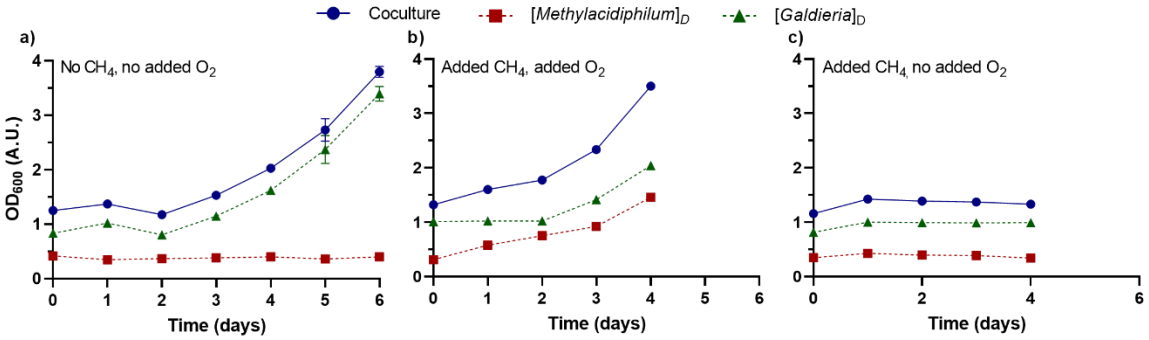


Figure 4.6. Growth of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture with different headspace concentrations a) Cocultures with no added CH_4 (to suppress methanotroph growth). b) Cocultures with daily replenishment of CH_4, CO_2, and O_2 to initial concentrations. c) Cocultures with no daily replenishment of O_2. Methanotroph and microalgae concentrations were calculated using the DSOF method. All cultures were cultivated at 45 °C, 150 rpm, and 40 µmol m^{-2} s^{-1} in 1 L gastight bottles on a shaking incubator. Cultures were grown in triplicate; error bars represent one standard deviation (n = 3).

I speculate that as O_2 accumulated in the media, coproporphyrin was re-absorbed (intracellularly) by *Galdieria* and subsequently synthesised into pigments, as in every replicate the substantial decrease in coproporphyrin was concurrent with a 1% drop in O_2 headspace concentration and was followed by the start of phycocyanin recovery. This is consistent with oxygen dependant oxidation of coproporphyrin III into the pigment precursor protoporphyrinogen IX as O_2 becomes available (Sarian *et al*., 2016). Anttila *et al.* (2011) found that *Paracoccus denitrificans* secretes coproporphyrin III for copper chelation when subjected to Cu-deprivation, and it can use the resulting complex as a source of copper. This implies that *P. denitrificans* possesses a transport system for the porphyrin complex uptake. The authors suggest a TonB-dependent heme receptor/transporter as responsible for porphyrin complex uptake, as they are known to transport tetrapyrroles, like coproporphyrin, through the outer and inner membranes to the cytoplasm (Anttila *et al.*, 2011). Additionally, Minoda *et al.* (2015) found that *Galdieria sulphuraria* could recover dissolved lanthanide and copper (II) ions and microscopically established that the metals accumulated inside the cells. The authors suggested that porphyrins could be responsible for this recovery, as they are

known to form complexes with Cu^{2+} (Ake & Gouterman, 1969) and lanthanides (Bulach, Sguerra, & Hosseini, 2012). Recovery of these metals was predominately observed within live semi-anaerobic (~ 99.8% N_2) heterotrophic cultures, but not in autotrophic (aerobic and semi-anaerobic), mixotrophic, or aerobic heterotrophic conditions, and it did not occur when using dead cells (Minoda *et al.*, 2015). As metal recovery occurred only under low O_2 conditions, their results are consistent with coproporphyrin excretion, metal sequestration, and subsequent uptake into the cells. Thus, it is possible that *Galdieria* sp. RTK37.1 is capable of coproporphyrin excretion, uptake, and subsequent pigment production when O_2 accumulates in the media. By day eight of coculture, coproporphyrin was no longer detectable, chlorophyll recovery was observed and O_2 began accumulating in the headspace. Following pigments recovery, *Galdieria* growth resumed, and pigments and O_2 accumulated as expected. Thus, the chlorosis in *Galdieria* caused by *Methylacidiphilum*-induced O_2 limitation was reversible, however a recovery period during which pigment biosynthesis resumed was required.

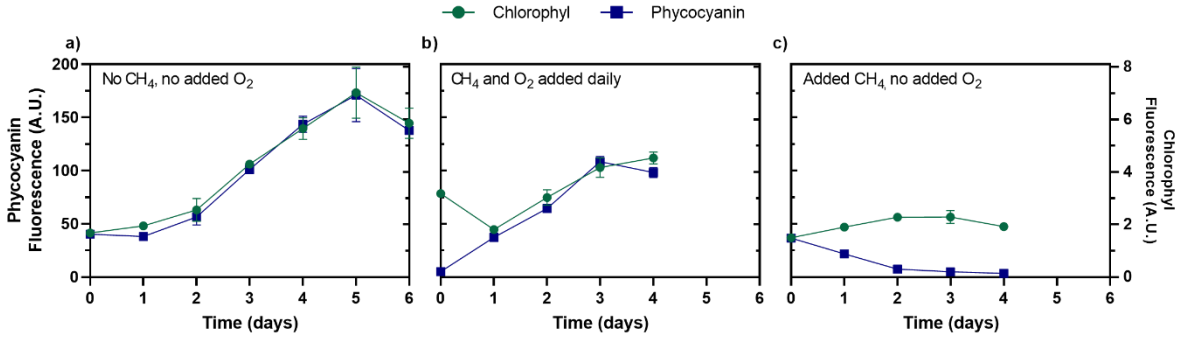


Figure 4.7. Pigment profiles for cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1. with different headspace concentrations a) Cocultures with no added CH_4 (to suppress methanotroph growth). b) Cocultures with daily replenishment of CH_4, CO_2, and O_2 to initial concentrations. c) Cocultures with no daily replenishment of O_2. All cultures were cultivated at 45 °C, 150 rpm, and 40 µmol $m^{-2}s^{-1}$ in 1 L gastight bottles on a shaking incubator. Cultures were grown in triplicate; error bars represent one standard deviation (n = 3).

I was unable to surmise the effect, if any, that coproporphyrin has on *Methylacidiphilum* sp. RTK17.1. Coproporphyrin has been found to function in cocultures as both growth promoter and inhibitor: Bhuiyan *et al.* (2016) described, in cocultures of *Leucobacter* sp. ASN212 and *Sphingopyxis* sp. GF9, that the coproporphyrin III secreted by *Sphingopyxis* sp. worked as growth factor for *Leucobacter* sp. in concentrations greater than 1.5 nM. At the same time, the compound was found to be toxic to *Sphyngopyxis*, and completely inhibited its growth at concentrations greater than 15 nM (Bhuiyan *et al.*, 2016). Also, the bacterium *Glutamicibacter arilaitensis* produces coproporphyrin III as a response to the presence of *Penicillium* sp. 12 in cheese rinds; in artificial cocultures, adding coproporphyrin III dihydrochloride slowed growth of both microorganisms at 100 and 200 µM concentrations. Inhibition at 200 µM was significantly greater for *Penicillium* (90% decrease, *p*-value < 0.001) than for *G. arilaitensis* (67% decrease, *p*-value < 0.001) (Cleary *et al.*, 2018), so coproporphyrin excretion may be a strategy to inhibit competitor growth (e.g. *Penicillium)*. It is possible coproporphyrin could inhibit *Methylacidiphilum* via nutrient sequestration. *Galdieria sulphuraria* is known to recover >90% of Cu^{2+} and lanthanide ions in solution at 0.5 ppm concentrations (Minoda *et al.*, 2015). This recovery may be mediated by coproporphyrin chelation of ions, which would make sense as coproporphyrin is used by *Paracoccus denitrificans* to scavenge copper

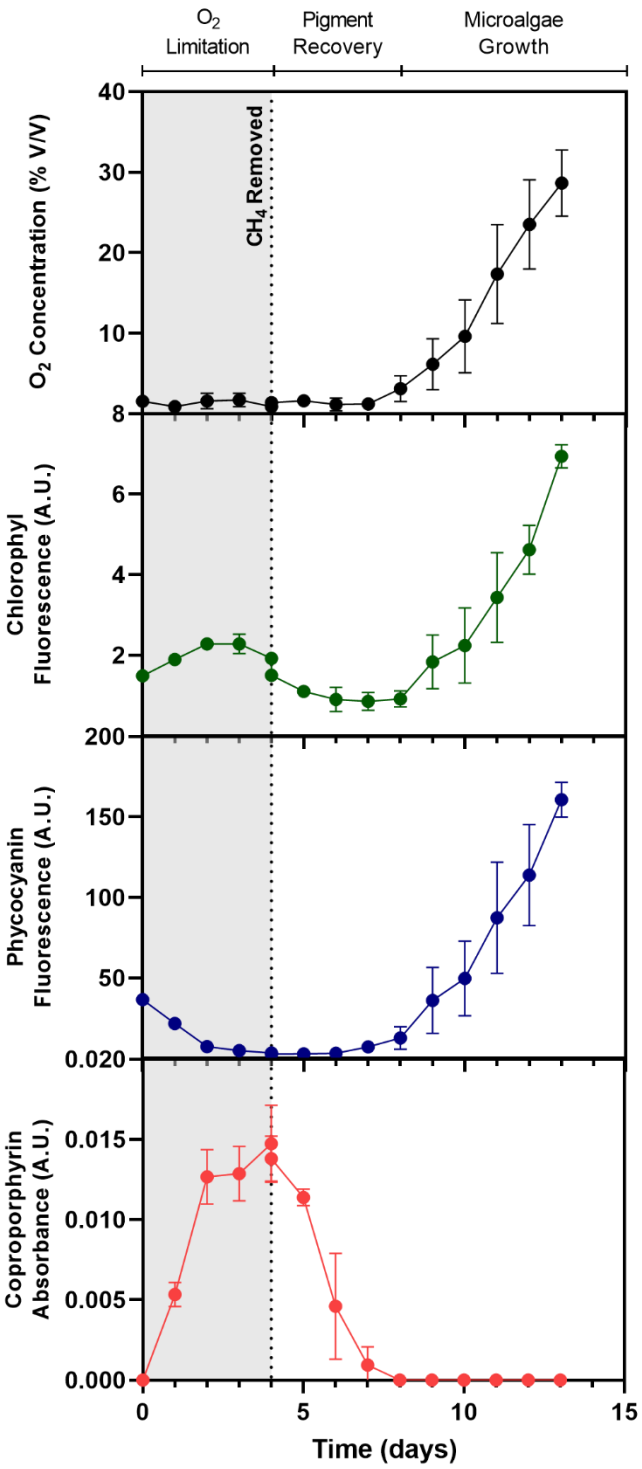


Figure 4.8. Chlorosis recovery of *Galdieria* sp. RTK37.1. Pigments (e.g. chlorophyl, phycocyanin), coproporphyrin, and O_2 profile in cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 with CH_4 initially added and later removed (Day four). Headspaces were subjected to vacuum, opened, and regassed on day 4 to remove CH_4. No O_2 was added at any point. Greyed-out area indicates the period where CH_4 was present. Coproporphyrin peak absorbance was measured by substracting the average of the optical densities at 390 and 410 from the 400 nm peak. Negative values calculated in this fashion were considered to be 0. Cocultures were incubated in 250 mL V4 media at 45 °C, pH 2.5, with 150 rpm agitation, and 40 µmol m^{-2} s^{-1} light intensity in 1 L gastight bottles. Cultures were grown in triplicate; error bars represent a standard deviation (n = 3).

(Anttila *et al.*, 2011). Growth of verrucomicrobial methanotrophs is dependent on lanthanides, as they are essential cofactors for the XoxF type methanol dehydrogenase that oxidises methanol to formate (Pol *et al.*, 2014). Additionally, Cu^{2+} is required for pMMO synthesis and function in methanotrophs (Semrau, DiSpirito, & Yoon, 2010). It is possible then than the coproporphyrin excreted by *Galdieria* sp. RTK37.1 could form complexes with these ions and inhibit *Methylacidiphilum* sp. RTL17.1 growth, however further research would be required to ascertain this.

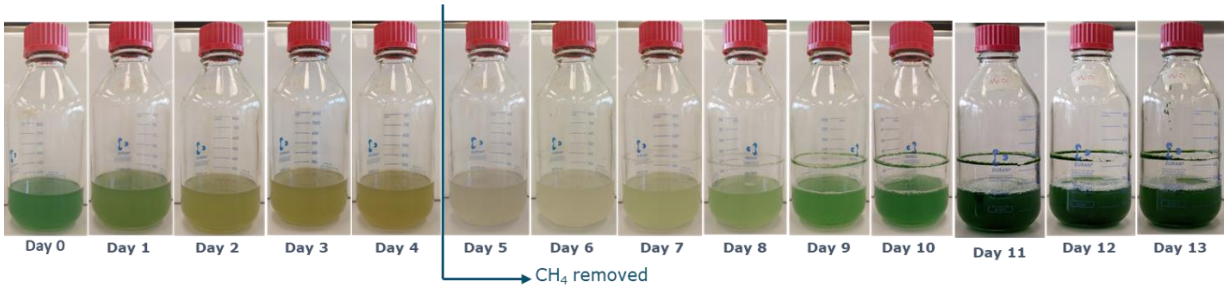


Figure 4.9. Colour development in batch cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 with CH_4 initially added and later removed. Headspace was subjected to vacuum, opened, and regassed on day 4 to remove CH_4. Cocultures were incubated in 250 mL V4 media at 45 °C, pH 2.5, with 150 rpm agitation, and 40 µmol m^{-2} s^{-1} light intensity in 1 L gastight bottles. Cultures were grown in triplicate, photographs taken every day of the same replicate.

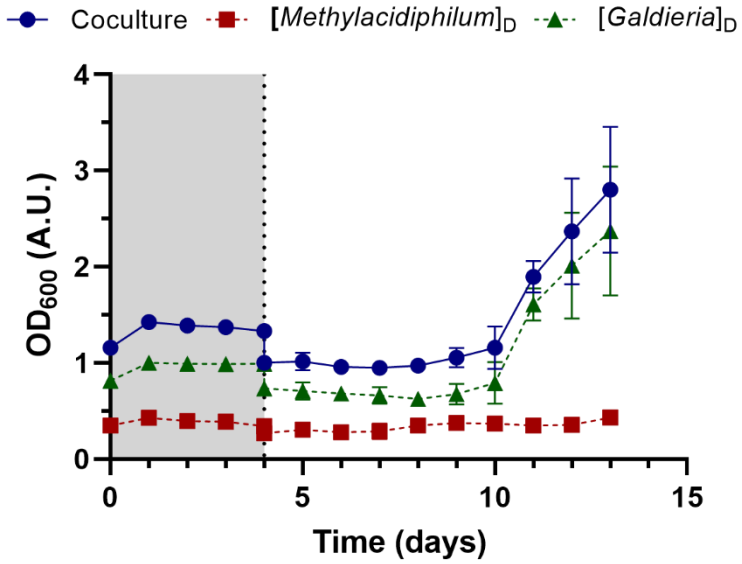


Figure 4.10. Batch coculture growth of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1. CH_4 (12.84 %) was initially added to the headspace and then removed (Day 4). Headspace was subjected to vacuum, opened, and regassed on day 4 to remove CH_4. Greyed-out area indicates the period where CH_4 was present. Coculture member concentrations were calculated using the DSOF method. Cocultures were incubated in 250 mL V4 media at 45 °C, with shaking (150 rpm), and 40 µmol $m^{-2}s^{-1}$ light intensity in 1 L gastight bottles. Cocultures were grown in triplicate; error bars represent one standard deviation (n = 3).

4.4 Conclusions

The relationship between *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1 in batch cocultures is dependent on O_2 availability and the relative concentrations of both microorganisms. When O_2 is limiting and *Methylacidiphilum* concentrations are relatively low, the methanotroph benefits from *Galdieria* oxygenic photosynthetic activity. However, as *Methylacidiphilum* concentration increases relative to *Galdieria*, its

high O_2 affinity disrupts *Galdieria* pigment biosynthesis which results in excretion of coproporphyrin III and subsequent chlorosis. Onset of chlorosis impairs oxygenic photosynthesis, which hinders pigment production further, and hence coculture growth. In coculture, the interactions between *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1 can therefore become deleterious if the relative concentration of *Methylacidiphilum* sp. RTK17.1 is too high (e.g., *Methylacidphilum*:*Galdieria* ratios ≥ 0.23). The threshold for chlorosis onset, however, is probably dependent on external O_2 and CH_4 supplies, as well as light intensity. Coproporphyrin excretion and chlorosis do not occur if O_2 is regularly replenished, and they are reversible if O_2 is allowed to accumulate. Our results seem to indicate that Galdieria sp. RTK37.1 requires a minimum concentration of O_2 in the media for adequate pigment (e.g. chlorophyll, phycocyanin) production, a behaviour to the best of our knowledge, not observed before in photoautotrophic algae. This is the first reported case of *Galdieria* spp. excreting coproporphyrin into the media during autotrophic growth, and the first case of it being directly caused by coculture with another microorganism. Based on its role in other cocultures, as well as in its chelating properties, coproporphyrin III has the potential to affect *Galdieria* and/or *Methylacidiphilum* growth, but more research would be required to investigate these effects. For this, chemostat coculture with continuous low O_2 concentrations would be useful, as it could potentially allow satisfying *Methylacidiphilum* sp. RTK17.1 O_2 requirements while at simultaneously triggering *Galdieria* sp. RTK37.1 coproporphyrin excretion.

Chapter 5. Continuous co-cultivation of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 reveals inhibition of the alga is O_2 concentration dependant

Abstract

My previous findings have demonstrated stable coculturing of the thermoacidophilic methanotroph, *Methylacidiphilum* sp. RTK17.1 and photoautotrophic alga *Galdieria* sp. RTK37.1. (Section 2.3). Furthermore, increased growth and methane (CH_4) oxidation rates in batch cocultures suggests *Methylacidiphilum* benefits from the oxygenic activity of *Galdieria* in a low oxygen (O_2) environment (Section 2.3.1). However, this beneficial interaction became deleterious whenever the relative mass ratio of *Methylacidiphilum* sp. RTK17.1 to *Galdieria* sp. RTK37.1 exceeded 0.18 (Section 4.3.1). Under these conditions, it was hypothesised that *Methylacidiphilum*'s high O_2 affinity caused a disruption to *Galdieria* pigment synthesis, resulting in coproporphyrin III excretion and subsequent chlorosis. Unravelling interspecies interactions within batch co-culture, however, is complicated as growth conditions change continuously with time. Thus, to better understand the effect different growth conditions have on *Methylacidiphilum-Galdieria* dynamics, continuous steady state coculturing (in parallel with axenic controls) was performed. For these steady state experiments, *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1 and a coculture of both were established in separate 1L bioreactors under a continuous feed gas (16 mL min^{-1}) that varied between 1% and 3% O_2. Results show the O_2 concentration and net uptake/production rates determine the nature of *Methylacidiphilum-Galdieria* interaction in coculture. In chemostat coculture at inlet O_2 < 2.1 % (D.O ≤ 0.198 ± 0.003 mg_{O2} L^{-1}), the interaction became inhibitory for *Galdieria*; with *Methylacidiphilum* sp. RTK17.1 benefiting from *Galdieria* sp. RTK.37.1, while simultaneously harming the microalgae by inducing O_2-limitation chlorosis. At inlet O_2 between 2.1-3.0 % (v/v) the relationship became neutral for *Galdieria*, *Methylacidiphilum* displayed faster growth, presumably due to greater O_2 availability allowing CH_4 to be oxidized further, without negatively affecting *Galdieria*. Findings from these experiments were then used to obtain a high biomass concentration in fed-batch (static liquid continuous gas flow) coculture in a 1L bioreactor. By continuously modifying growth conditions (e.g. light, O_2, NH_4^+) a final biomass concentration of 2.73 g_{DW} L^{-1} following 38 days incubation was obtained. This represents the most concentrated *Methylacidiphilum-Galdieria* coculture reported to date. Overall, results of these chemostat coculture experiments allow us to better understand how O_2 concentration affects *Methylacidiphilum-Galdieria* interactions. Not only will these results help facilitate conceptualization and bioprocess design to further improve *Methylacidiphilum-Galdieria* coculture performance, but they expand our knowledge of methanotroph-algae interactions.

5.1 Introduction

Methanotroph-photoautotroph cocultures have attracted attention in recent years as ways to recover energy and capture carbon from both CH_4 and CO_2 (Badr *et al.*, 2019; van der Ha *et al.*, 2011). They have also been studied for their potential in producing valuable products including biodiesel (Li *et al.*, 2022), bioplastics (van der Ha *et al.*, 2012), and single cell proteins (Rasouli *et al.*, 2018), as well as for the recovery of nutrients from industrial wastewater (Rasouli *et al.*, 2018; Roberts *et al.*, 2020; van der Ha *et al.*, 2011). In this regard, cocultures of the thermoacidophilic *Methylacidiphilum* sp. RTK17.1 (an aerobic

methanotrophic bacterium) and *Galdieria* sp. RTK37.1 (an oxygenic photoautotrophic microalga) are interesting, as they are capable of growing under industrially relevant conditions: hot temperatures due to the bubbling of hot flue gases, high CO_2, and acidic pH (Carere *et al.*, 2017; Varshney *et al.*, 2015; Vítová *et al.*, 2016).

My previous findings show that cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 are stable, and that *Methylacidiphilum* benefits from *Galdieria* presence in a low oxygen (O_2) environment. Under these conditions increased growth (29 %) and methane oxidation (300 %) (*p*-values < 0.05 and < 0.001 respectively) in batch cocultures were observed (Section 2.3.1). However, the beneficial interaction became deleterious whenever the relative mass ratio of *Methylacidiphilum* sp. RTK17.1 to *Galdieria* sp. RTK37.1 exceeded 0.18. It was hypothesised that *Methylacidiphilum's* high O_2 affinity disrupted *Galdieria's* O_2-dependent pigment synthesis, resulting in coproporphyrin III excretion and chlorosis (Section 4.3.2). Coproporphyrin has previously been reported to function as both a growth promoter (Bhuiyan *et al.*, 2016) and a growth inhibitor (Cleary *et al.*, 2018) in cocultures, and as such its role in *Methylacidiphilum-Galdieria* interactions remained uncertain.

The relationship status between partners in cocultures is known to change in response to environmental factors (Goers *et al.*, 2014; Hoek *et al.*, 2016; Hom *et al.*, 2015). For example, in two non-mating engineered strains of *Saccharomyces cerevisiae,* each modified to be deficient in the synthesis of an essential amino acid (leucine or tryptophan) and over-produce the amino acid required by its partner, Hoek *et al.* (2016) reported that modifying amino-acid concentrations (from low to high), resulted in the pair exhibiting (in order): collapse of the mutualism, obligatory mutualism, obligatory/facultative mutualism, facultative mutualism, parasitism, competition, and competitive exclusion. In microalgae-bacteria interactions nutrition exchange tends to be the base of mutualistic relationships, most commonly microalgae provide O_2 and organic substances through photosynthesis, and bacteria produce CO_2 and inorganic substances through respiration that sustain microalgal growth (Zhang *et al.*, 2020). However, these mutualisms can develop into various degrees of antagonisms depending on factors such as light intensity, mixing, and temperature (Chen *et al.*, 2009; Hom *et al.*, 2015).

In photoautotroph-methanotroph cocultures, possible changes in partner interactions have not been widely studied. In most reported cases, when O_2 is limiting, methanotroph biomass production and/or CH_4 oxidation is enhanced by microalgae growth. This is the case for cocultures of the microalga *Scenedesmus* sp. with the methanotroph *Methylocystis parvus* (van der Ha *et al*., *2012)* and the microalga *Chlorella sorokiniana* with the methanotroph *Methylococcus capsulatus* (Rasouli *et al.*, 2018). For cocultures of the cyanobacteria *Synechococcus* PCC 7002 with the methanotroph *Methylomicrobium alcaliphilum* 20z, Hill *et al.* (2017) reported than in chemostat coculture, *M. alcaliphilum* biomass production rates were influenced by the input of light; as all O_2 was supplied via *Synechococcus* photosynthetic activity. However, in batch cocultures of the same pair, Badr *et al.* (2020) reported that the photoautotroph:methanotroph ratio did not affect growth, as the limiting factor was CH_4 mass transfer rather than O_2 availability. Furthermore, there are also cases of microalgae negatively affecting methanotrophs in coculture: Ruiz-Ruiz *et al.* (2020) found that in cocultures with excess O_2, overabundance of the green microalgae *Scenedesmus obtusiusculus* slowed the observed CH_4 degradation rates 27 - 43 % for an alkaliphilic methanotrophic

bacterium consortium. The CH_4 degradation was dependant on the relative ratio of S. *obtusiusculus* and the methanotroph consortium. This was attributed to the microalga alkalizing the media, as it consumed CO_2, beyond the optimum pH for the bacterium (Ruiz-Ruiz *et al.*, 2020). An interesting O_2 interaction was also described by Li *et al.* (2022), who found that culturing the mixotrophic microalga *Scenedesmus obliquus* with the methanotroph *Methylocystis bryophila* diminished chlorophyll concentration but increased cell density of the microalga. Presence of the bacterium induced decreased expression of genes related to the Calvin Benson Bassham Cycle and photosynthetic activity in the microalga. It was found that the organic carbon released by *M*. *bryophila* triggered a switch from autotrophic to mixotrophic growth in *S. obliquus*, which explains the coincident increase in biomass. Thus, the coculture pair dynamically adapted to O_2 availability. At low O_2 concentrations the microalga tended to grow autotrophically and the methanotroph would convert CH_4 into CO_2, while at high O_2 concentrations, heterotrophic algal growth would dominate, the methanotroph would not grow, and release organic matter instead of CO_2 (Li *et al.*, 2022).

The vast majority of reported photoautotroph-methanotroph cocultures have been conducted in batch or semi-batch conditions (Badr *et al.*, 2022; Li *et al.*, 2022; Rasouli *et al.*, 2018; van der Ha *et al.*, 2011). However, batch coculturing complicates the analysis of potential interspecies interactions, as conditions change continuously with time. Thus, to better understand the effect different growth conditions have on *Methylacidiphilum-Galdieria* dynamics, continuous steady state coculturing (in parallel with axenic controls) was performed. Other than my previous study (Chapter 2), the only reported photoautotroph-methanotroph steady state coculture was conducted by Hill *et al.* (2017) with *Synechococcus* PCC 7002 and *M. alcaliphilum* 20z. However, only a single steady state was analysed, and no axenic controls were performed. Chemostats can help understand interspecies interactions under defined stable environmental conditions (Beliaev *et al*., 2014) therefore, by understanding how these conditions affect coculture performance, bioprocesses can be more easily optimised and scaled up. Hence the aim of this chapter is to further study the interactions between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in low O_2 environments in steady state continuous coculture, and to use the results to obtain a high concentration fed-batch (static liquid, continuous gas flow) coculture.

5.2 Materials and Methods

5.2.1 Chemostat reactors initialization

Steady state culturing of *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1, and a coculture of both microorganisms was established using three identical 1 L bioreactors (BioFlo 110; New Brunswick Scientific, Edison, NJ, USA). Cultures were incubated at 45 °C, with a 900 mL working volume, and V4 media (Appendix A) at pH 2.5. Two Rushton type impellers were used in each reactor, and agitation was maintained at 400 rpm for *Galdieria* and 250 rpm for *Methylacidiphilum* and the coculture. Dissolved O_2 was monitored using an InPro 6810 Polarographic Oxygen Sensor (Mettler-Toledo, Columbus, OH, USA). pH was monitored with a Polilyte Plus pH Sensor (Hamilton Company, United States). Custom gas mixtures were prepared in compressed gas cylinders and supplied at 16 mL min^{-1} using mass flow controllers (El-flow, Bronkhorst, Netherlands). Feed gas was filter sterilised before entering the reactors using in-line 0.22 µm hydrophobic filters (MicroScience, Australia). Inlet gas composition for *Methylacidiphilum* sp. RTK17.1 (R1) and the coculture (R2) bioreactors was approximately 2.1 % O_2, 1.1 % CH_4, 74 % CO_2, balance N_2,

(all v/v). For the *Galdieria* sp. RTK37.1 bioreactor (R3), feed gas composition was 3.0 % O_2, 64 % CO_2, balance N_2. Light was supplied to the axenic microalgae and coculture reactors (R3 and R2) via warm white LED strips attached to the walls of the reactor. Light intensity was adjusted to 100 µmol photons m^{-2} s^{-1} measured at the centre of the empty reactor with a LI-250A Light Meter (LI-COR, Nebraska, United States). Optical density at 600 nm was routinely measured using an Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom). To initiate axenic cultures (R1 and R3), reactors were inoculated to OD_{600} 0.1 A.U. with the corresponding microorganism and grown in batch mode until an OD_{600} of 1.0 A.U. was reached; after which the inlet and effluent peristaltic pumps (Cole Palmer Instruments, United States) were activated. For the coculture, the reactor (R2) was inoculated to 0.1 A.U. OD_{600} of *Galdieria* sp. RTK37.1, grown in batch until OD_{600} = 1.0 A.U. and then the pumps were started. After achieving steady state, < 1 mL *Methylacidiphilum* sp. RTK17.1 (OD_{600} 1.20 A.U.) was added to the reactor, and a new steady state was achieved. Bioreactor working volume was maintained at 900 mL by automatic regulation of the culture level with a scavenging/effluent pump. For the axenic methanotroph (R1) and the coculture (R2), media was supplied, and spent broth withdrawn at a constant flow rate of 150 mL day^{-1} (D = 0.167 day^{-1}). For the microalgal chemostat (R3) a flowrate of 250 mL day^{-1} was used (D = 0.278 day^{-1}). For all reactors 3 mL liquid and 40 mL inlet and outlet gas samples were harvested daily using aseptic technique for biomass (OD_{600}) and gas measurements. Systems were considered ‘steady state’ when there was less than a 5% OD_{600} variation over a three-day period.

5.2.2 Chemostat Coculture Experiments

To study the effect that O_2 concentration has on steady state cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1, inlet feed gas O_2 concentration was varied between 1% and 3% (v/v). For these experiments, the liquid flowrate and agitation rate for all chemostat reactors was set to 250 mL day^{-1} (D = 0.278 day^{-1}) and 400 rpm. Inlet feed gas flowrate was 16 L min^{-1} for all reactors. Custom gas mixtures with the desired O_2 concentration were prepared in compressed gas cylinders and supplied to the reactors. In total, four different inlet O_2 concentrations were used: 1.0, 1.6, 2.1, and 3.0 % (v/v). The feed gas concentrations for all experimental conditions are shown in Table 5.1. The remaining experimental conditions were as described in section 5.2.1 . For all reactors, 2 mL liquid and 40 mL inlet and outlet gas samples were collected daily using aseptic technique for biomass (OD_{600}), pigment fluorescence, absorption spectra, and gas measurements. For each of the inlet O_2 experimental conditions, after achieving steady state, 200 mL of outlet broth was collected to determine biomass concentration gravimetrically. Also, to determine protein content and amino acid profiles, biomass was collected daily and stored at 4 °C. After 1 L of broth had accumulated, it was centrifuged at 12,000 rpm for 15 minutes. Supernatants were then discarded, and the resulting biomass pellets were stored at -20 °C until needed.

5.2.3 Fed-batch Coculture (Static-liquid, continuous gas)

To investigate the coculture biomass productivity limitations of the existing bioreactor configuration, a high concentration batch coculture of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 was performed. For this experiment, the coculture chemostat (R2) was switched to 2.1% inlet O_2 feed gas and allowed to reach steady state. All experimental conditions were set as described in Section 5.2.2 . Once steady state was reached, the inlet and effluent pumps were turned off to allow batch growth. Liquid (2 mL) and inlet

and outlet gas samples (40 mL) were collected daily using aseptic technique. Gas samples were analysed for gas concentrations. Liquid samples were analysed for total biomass concentration (OD_{600}), proportional biomass concentrations (via DSOF), and pigment content (e.g. chlorophyll, phycocyanin, coproporphyrin). In addition, 10 mL aliquots were routinely collected from the reactor (with V4 media replacement) to measure biomass concentration gravimetrically. To avoid nitrogen limitation, 10 mL of ammonium chloride (40 g L^{-1}) were added when total OD_{600} reached 3.0, 6.0 or 9.0 AU. To avoid chlorosis, gas flowrate and/or light intensity was increased if O_2 outlet concentration decreased below 1.6%, coproporphyrin excretion was detected or coculture growth stopped. Cultivation was terminated upon the onset of stationary phase.

Table 5.1. Inlet feed gas CO_2, CH_4, and O_2 concentrations for the chemostat cultures and cocultures.

Nominal Inlet O_2 Concentration	Inlet gas concentration[a] (% v/v)							
	Reactor R1[b]			Reactor R2[c]			Reactor R3[d]	
	O_2	CH_4	CO_2	O_2	CH_4	CO_2	O_2	CO_2
1.0	0.97±0.01	1.02±0.01	74.64±0.22	0.96±0.01	1.03±0.03	74.43±0.19	1.01±0.04	72.07±0.31
1.6	1.64±0.03	1.08±0.01	73.85±0.29	1.58±0.04	1.09±0.02	73.79±0.21	1.60±0.01	71.84±2.48
2.1	2.05±0.02	1.09±0.01	72.97±0.14	2.07±0.01	1.09±0.01	72.97±0.14	2.06±0.03	74.49±1.50
3.0	3.21±0.27	1.03±0.01	74.08±0.14	2.99±0.01	1.02±0.01	74.57±0.73	3.07±0.08	73.30±0.78

[a]Balance N_2, concentrations are triplicate measurements with a standard deviation (n=3).
[b]R1 refers to axenic *Methylacidiphilum* sp. RTK17.1 chemostat culture.
[c]R2 refers to *Methylacidiphilum* sp. RTK17.1 and *Galdieria* RTK37.1 sp. chemostat coculture.
[d]R3 refers to axenic *Galdieria* sp. RTK37.1 chemostat culture.

5.2.4 Analytical procedures

Optical density at 600 nm was routinely measured using an Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom). To differentiate the contribution of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 to the coculture OD_{600}, the DSOF method (Section 3.2.2) was used.

Variation in pigments in coculture were monitored by autofluorescence. For these measurements, 200 µL samples were transferred into black, flat bottom (chimney well) 96-well microplates (Greiner Bio-One, Austria). Fluorescence was then measured at 670 nm emission (590 nm excitation) for phycocyanin (Gregor & Maršálek, 2005) and at 670 nm emission (485 nm excitation) for chlorophyll (Gregor & Maršálek, 2005) in a in a plate reader spectrophotometer (Varioskan Lux, Thermo Scientific, United States). For absorption spectra, 200 µL samples were transferred into clear 96-well microplates (Greiner Bio-One, Austria) and optical density was measured for wavelengths between 300 to 800 nm in 1 nm increments. To quantify the variation in coproporphyrin, the average of the optical density values at 390 and 410 was subtracted from the 400 nm peak value. Negative values calculated in this fashion were considered 0.

Biomass concentrations were measured gravimetrically. For chemostat reactors, 50 mL aliquots from the collected broth were centrifuged at 12,000 rpm for 15 minutes. Supernatants were then discarded, and the pellets were resuspended in deionized water and centrifuged again for 15 minutes. Supernatants were discarded and the pellets were transferred into pre-weighed aluminium dishes and left to dry 12 hours within a 95 °C oven. The aliquot biomass weight was calculated by difference. Technical triplicates were measured. For the fed-batch coculture, 10 mL aliquots were collected from the reactor, and vacuum-filtered onto pre-weighted 0.22 µm nylon membrane filters (MicroScience, Australia). The filters were then washed 3 times by vacuum-filtering 10 mL of deionized water, and dried 12 hours at 95 °C.

Gas samples were analysed for CO_2, CH_4, N_2, and O_2 concentrations using a 490 micro GC equipped with a thermal conductivity detector (Agilent Technologies, United States), equipped with Molecular Sieve 5A with a heated injector (50 °C, back-flush at 5.10 s, column at 90 °C, 150 kPa), a PoraPak Q column with a heated injector (50 °C, no back-flush, column at 70 °C, 50 kPa) and a 5CB column with a heated injector (50 °C, no back-flush, column at 80 °C, 150 kPa).

Nutritional characterization of biomass (ash, crude protein, and amino acid profiles) was performed by the Massey University Nutrition Laboratory according to the official methods of analysis of the Association of Official Analytical Communities international (AOAC, 2005). Ash content was determined by the furnace method (AOAC method 9442,05), total crude protein was determined via the Dumas method (AOAC method 968.06). Amino acid profile determination of acid-stable residues was performed via reverse-phase high performance liquid chromatography (HPLC) separation using AccQ derivatization of biomass (60–140 mg) samples following oxidization with performic acid and hydrolysis with hydrochloric acid as described in AOAC method 994.12 (AOAC, 2005). Cysteine and methionine contents were not measured.

All Statistical analysis were performed using Prism Graphpad 9.4.1. Unless stated otherwise, Two-factor ANOVA (alpha = 0.05) tests were used, with Sidak method for multiple mean comparisons between columns (simple effects within rows) or rows (simple effects within columns). Growth rates were calculated by fitting the appropriate concentration data to the exponential growth equation model in Prism Graphpad 9.4.1 ($Y = Y_0 e^{\mu_{max} t}$, where Y is the concentration in A.U., Y_0 is the concentration at time zero in A.U., μ_{max} is the specific growth rate in h^{-1}, and t is time in h).

5.3 Results and discussion

This study was the first to explore the interactions between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in low O_2 environments using continuous coculturing techniques. To investigate the impact of O_2 concentration on coculture interactions, inlet feed gas O_2 concentration was varied between 1% and 3% (v/v) in parallel axenic and coculture steady state bioreactors. For the *Methylacidiphilum* sp. RTK17.1 and coculture chemostats (R1 and R2 respectively) increasing feed gas O_2 concentration from 1.0 to 2.1 % (v/v) increased the steady state biomass concentration 83 % and 72 % (*p*-values < 0.001, Figure 5.1) respectively. This observation is consistent with our previous observation that O_2 limitation in the 1.0-2.1 % (v/v) range for both *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1 in batch coculture (Sections 2.3.1 and 4.3.2). Further incremental addition to inlet O_2 concentration had no significant effect on the steady state biomass concentration in either chemostat (*p*-value > 0.05); which suggests O_2 was not limiting at inlet concentrations > 2.1% (v/v). For the coculture at 3.0 % O_2, a maximal steady state biomass concentration of 0.263 ± 0.02 g_{DW} L^{-1} was achieved. This is relatively low compared to the chemostat coculture of the cyanobacterium Synechococcus 7003 and *Methylomicrobium alcaliphilum* (D = 0.3 h^{-1}), which maintained a steady state concentration of 0.68 ± 0.01 g_{DW} L^{-1} (Hill *et al.*, 2017). For all inlet O_2 conditions tested, the *Galdieria* sp. RTK37.1 chemostat (R3), was not significantly affected (the slope of a linear regression was not significantly different from 0, *p*-value > 0.05). Thus, even at 1.0 % inlet O_2, axenic *Galdieria* did not become O_2-limited, which suggests *Methylacidiphilum* sp. RKT17.1 activity is responsible for inducing O_2 limitation in coculture chemostats (R2).

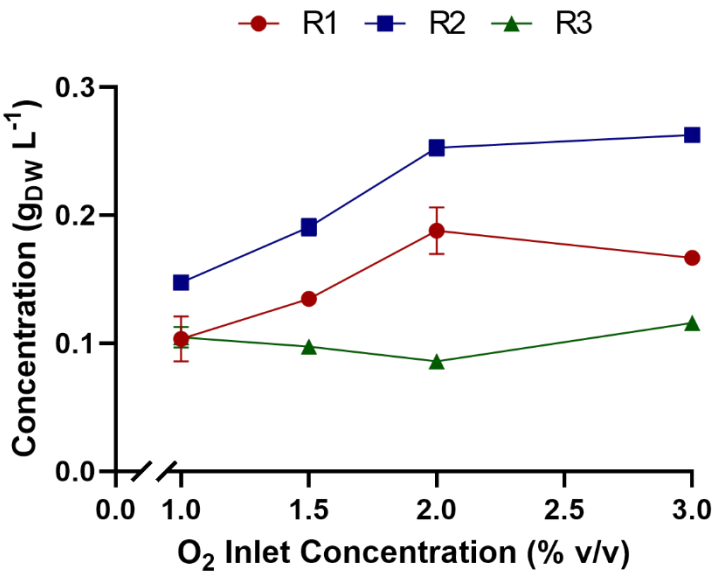


Figure 5.1. Steady state biomass concentrations at different inlet O_2 concentrations for *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 chemostat cocultures and axenic controls. R1 is the *Methylacidiphilum* sp. RTK17.1 chemostat, R2 is the coculture chemostat, and R3 is the *Galdieria* sp. RTK37.1 chemostat. All cultures were grown in identical 1 L stirred tank reactors at 45 °C, pH 2.5, 400 rpm, D = 0.278 day^{-1}, 900 mL working volume and 100 µmol m^{-2} s^{-1} measured at the middle of the empty reactor. Feed gas was supplied continuously at a 16 mL min^{-1} rate. Values correspond to steady state measurements. The average value of triplicate measurements (n=3) is shown with error bars representing one standard deviation.

I next quantified *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 concentrations in the coculture chemostat (R2), using the DSOF method, to further understand their individual response to O_2 limitation. A 2-way ANOVA was performed to compare *Methylacidiphilum* sp. RTK17.1 concentration within axenic (R1) and coculture (R2) chemostats (Figure 5.2a). Results indicate both the O_2 inlet concentration and *Galdieria* presence had a significant effect (*p*-value < 0.0001) on the steady state biomass concentration of *Methylacidiphilum* sp. RTK17.1, with 69.75% of the observed variation attributed to inlet O_2 concentration and 18.85% due to *Galdieria* presence/absence. Within the axenic chemostat (R1), growth was limited by O_2 at inlet concentrations between 1.0-2.1 % (Table F.1). Further increasing O_2 concentration caused a small but significant decrease in methanotroph OD_{600} (from 0.27 to 0.25 A.U.), which I attribute to the small dilution of inlet CH_4 concentration in the 3.0% O_2 inlet gas mixture (Table 5.1). *Methylacidiphilum* sp. RTK17.1 concentration in coculture was significantly greater than in axenic cultures for all nominal inlet O_2 concentrations (Table F.2). This observation can be attributed to the additional O_2 produced by *Galdieria* growth partially countering the effect of O_2 limitation. In coculture (R2), *Methylacidiphilum* concentrations only significantly increased when the O_2 inlet was increased from 1.0 to 1.6 % (Table F.3), which suggests that at inlet $O_2 \geq 1.6\%$ *Galdieria* was able to provide sufficient O_2 for the methanotroph to overcome this limitation. This was further evidenced with the coculture O_2 consumption rates (Figure 6.4b), which are constant between 1.6 and 3.0 % O_2 inlet. This also explains why the difference in *Methylacidiphilum* concentrations between reactors diminished as O_2 concentration increased (a 24% difference at 1.0% O_2 reduces to an 8.7% difference at 3.0% O_2), as the extra O_2 would be most beneficial when O_2 was most constrained. As in the axenic culture, I attribute the small decrease in methanotroph concentration at 3.0% inlet O_2 to the small CH_4 concentration differences between gas mixtures.

As for *Methylacidiphilum* sp. RTK17.1, a 2-way ANOVA was also performed to compare steady state *Galdieria* sp. RTK37.1 biomass concentrations within axenic and coculture chemostats (Figure 5.2b). Results indicate the O_2 inlet concentration (43.16% variation), *Methylacidiphilum* presence (12.44% variation), and the interaction between both factors (41.93% variation) had a significant effect (*p*-value < 0.0001) on microalgae concentration. *Galdieria* sp. RTK37.1 in coculture (R2) was considerably less concentrated compared to the axenic chemostat (R3) for O_2 inlet concentrations ≤ 1.6% (Table F.4). These results are consistent with observed pigment and coproporphyrin contents (Figure 5.3). At O_2 inlet concentrations ≤ 1.6%, steady state *Galdieria* OD_{600} values in the chemostat were less than their axenic counterparts, while chlorophyll and phycocyanin contents similarly diminished. Also, at these O_2 concentrations, coproporphyrin was detected. Coproporphyrin is an intermediate in the biosynthesis of both phycocyanin and chlorophyll (Stadnichuk *et al.*, 1998), and it is secreted by *Galdieria* spp. as a response to O_2 limitation (Sarian *et al.*, 2016; Zhu *et al.*, 2022) which leads to pigment degradation (chlorosis) (Section 4.3.1). In this study, the observed rates of O_2 uptake by *Methylacidiphilum* were 2.1 to 4.6-fold faster than the rates of photosynthetic O_2 production by *Galdieria* (Figure 5.4). Consequently, more O_2 was consumed by the coculture than what was produced. This, in combination with *Methylacidiphilum*'s exceptionally strong oxygen affinity (K_s = 0.7 µM) (Pol *et al.*, 2007), suggests that the methanotroph is able to rapidly scavenge the majority of O_2 produced phtosynthetically. Thus, at inlet O_2 concentrations < 1.6 % (v/v, D.O. ≤ 0.198 ± 0.003 mg_{O2} L^{-1}) chlorosis was triggered; as evidenced by chlorophyll (Figure 5.3a) and phycocyanin (Figure 5.3b) fluorescence diminishing, and by coproporphyrin (Figure 5.3c) presence in the media. As pigments degraded, photosynthetic activity, and in consequence, microalgae growth was slowed down.

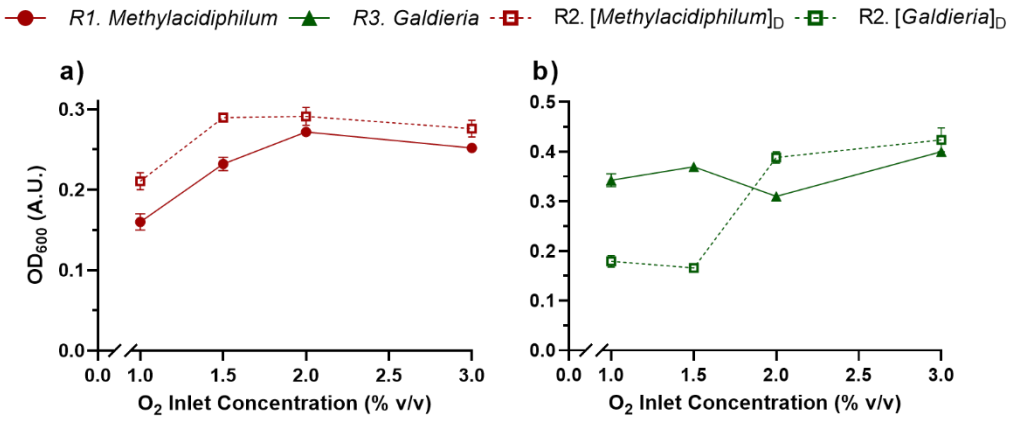


Figure 5.2. *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 steady state biomass concentrations at different inlet O_2 concentrations in chemostat cocultures and axenic controls. a) *Methylacidiphilum* sp. RTK17.1 concentration in coculture chemostat (R2) compared to its concentration in the axenic chemostat (R1). b) *Galdieria* sp. RTK37.1 concentration in coculture chemostat (R2) compared to its concentration in the axenic chemostat (R3). Methanotroph and microalgae abundance in cocultures were calculated using the DSOF method. All cultures were grown in 1 L stirred tank reactors at 45 °C, pH 2.5, 400 rpm, D = 0.278 day^{-1}, 900 mL working volume and 100 µmol m^{-2} s^{-1} measured at the middle of the empty reactor. Feed gas was supplied continuously at 16 mL min^{-1}. Values correspond to steady state measurements. The average value of five measurements (n=5) is shown with error bars representing one standard deviation.

There were several noteworthy differences to coproporphyrin excretion in these chemostats experiments compared to the O_2 limited batch cocultures in serum bottles (Section 4.3.1). Firstly, coproporphyrin

absorbance in the chemostats was ~ 10-20% of the maximum values obtained for the batch experiments. This is probably a result of *Galdieria* biomass concentration differences between systems: the batch cocultures had a starting *Galdieria* OD_{600} = 0.88 A.U., while chemostat steady state values were ~ 0.17 A.U. It is important to note, however, that steady state coproporphyrin values almost doubled (from 0.0019 A.U. to 0.0039 A.U., p-value < 0.0001) when decreasing O_2 inlet from 1.6 to 1.0 %. This indicates that more coproporphyrin was excreted per cell with worsening O_2 limitation. This has also been reported by Zhu *et al.* (2022) for cocultures of mixotrophic *Galdieria sulphuraria* UTEX 2919 with a bacterial community from industrial wastewater. In this coculture the added O_2 consumption from the microbial community resulted in increased coproporphyrin excretion, slower growth rates and lesser biomass titre compared to *Galdieria* grown within sterilized media, where O_2 limitation would be less severe (Zhu *et al.*, 2022). It is possible then that further decreasing O_2 inlet concentration could lead to *Galdieria* growth stopping or slowing down sufficiently ($D > \mu$) to be washed out of chemostat coculture. Interestingly, in chemostat coculture biomass and O_2 production was observed concurrent to coproporphyrin excretion. I have previously established in batch experiments that when pigment synthesis stops in *Galdieria* sp. RTK37.1 phycocyanin starts degrading (Section 5.3). This means then, since steady state was achieved, both pigment synthesis and coproporphyrin excretion were occurring at the same time. The biochemical basis for why low O_2 inhibits pigment synthesis is that the oxygen-dependent oxidoreductase (coproporphyrinogen III oxidase), HemF (E.C. 1.3.3.3), which catalyses the oxidation of coproporphyrinogen III into protoporphyrinogen IX (the precursor for chlorophyll and phycocyanin), is completely inhibited by anaerobic conditions (Sarian *et al.*, 2016; Skotnicová *et al.*, 2018). Nevertheless, an oxygen-independent oxidoreductase, HemN (1.3.99.22), exists but it is found mostly on prokaryotic organisms (Sarian *et al.*, 2016; Skotnicová *et al.*, 2018). Homologues for genes encoding HemN have previously been identified in the genome of *Galdieria sulphuraria* 074G, and chlorophyll production under O_2 limitation has been reported for the strain (Sarian *et al.*, 2016). However, my previous experiments found no evidence of chlorophyl, phycocyanin, or O_2 production in *Galdieria* sp. RTK37.1 under O_2 limitation (Section 5.3). This suggests that under O_2 limitation *Galdieria* sp. RTK37.1: a) HemF is still capable of oxidising a fraction of the coproporphyrinogen III pool, while the rest is excreted to the media, and b) the relative extent of both processes is dependent on O_2 concentration. The mechanism by which coproporphyrin is excreted has not been described, but our current findings and the apparent re-absorption of coproporphyrin (Section 4.3.2), leads me to speculate that for *Galdieria* sp. RTK37.1 such mechanisms may be dependent on dissolved O_2 concentration. Finally, in chemostat coculture, I was able to trigger coproporphyrin production while at the same time continuously supplying O_2 to the system, so that *Methylacidiphilum* growth was possible. As *Methylacidiphilum* concentration was greater in coculture at all inlet O_2 concentrations, there is no evidence that coproporphyrin inhibited its growth. It has been reported that coproporphyrin completely inhibits *Sphingopyxis* sp. GF9 at concentrations > 15 nM (Bhuiyan *et al.*, 2016), and that coproporphyrin III dihydrochloride at concentrations > 100 µm slows growth of *Penicillium* sp. 12 and *Glutamicibacter arilaitensis* (Cleary *et al.*, 2018). It is still possible, then, that greater coproporphyrin concentrations could negatively impact the methanotroph.

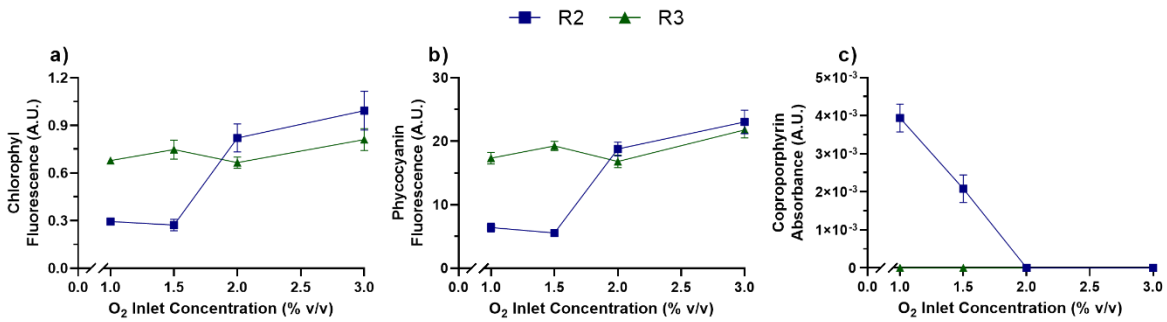


Figure 5.3. Pigments and coproporphyrin steady state contents for *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 chemostat cocultures and axenic controls. a) Chlorophyll fluorescence, b) Phycocyanin fluorescence, and c) Coproporphyrin peak absorbance was measured by substracting the average of the optical densities at 390 and 410 from the 400 nm peak. Negative values calculated in this fashion were considered to be 0. All cultures were grown in 1 L stirred tank reactors at 45 °C, pH 2.5, 400 rpm, D = 0.278 day^{-1}, 900 mL working volume and 100 µmol m^{-2} s^{-1} measured at the middle of the empty reactor. Feed gas was supplied continuously at a 16 mL min^{-1} rate. All values are steady state averages of 5 consecutive measurements (n=5). Error bars represent one standard deviation.

Steady state dissolved oxygen (D.O.) (Figure 5.4a) values were significantly different between reactors (*p*-value < 0.001) and changed with increasing inlet O_2 concentrations (*p*-value < 0.0001). Interestingly, there was less dissolved O_2 in the coculture (R2) than in the axenic *Methylacidiphilum* sp. RTK17.1 chemostat (R1) for all inlet O_2 concentrations. One might expect D.O. to be more concentrated in the coculture, as both: 1) *Galdieria* is supplementing O_2 via photosynthetic activity and 2) the coculture consumed significantly lesser O_2 compared to the axenic *Methylacidiphilum* chemostat (*p*-value < 0.001) (Figure 5.4b). However, as already stated, the additional photosynthetically produced O_2 supported faster *Methylacidiphilum* growth, which consequently increased steady state methanotroph biomass concentrations in the reactor leading to depleted O_2 in the media. A similar behaviour has already been observed in mixed cultures of mixotrophic *Galdieria sulphuraria* and bacteria within ammonium-rich wastewater, where D.O. in fed-batch *Galdieria* cultures was consistently lesser (and biomass concentrations greater) than within the mixed cultures (Zhu *et al.*, 2022). In the axenic *Galdieria* sp. RTK37.1 chemostat (R3), D.O. increased linearly with inlet O_2 concentration. This observation is expected as *Galdieria* was not O_2 limited, and therefore increasing O_2 inlet concentration had no effect on its net volumetric (Figure 5.4c) and specific production rates (Figure 5.4d). For both axenic *Methylacidiphilum* (R1) and coculture (R2) chemostats, relatively little difference in D.O. was observed at inlet O_2 < 2.1 %. At these concentrations, both systems were O_2 limited and increasing O_2 supply resulted in enhanced biomass production. In contrast, at O_2 inlet concentrations ≥ 2 % cultures were not O_2 limited and D.O. increased linearly, as was observed in the *Galdieria* chemostat (R3).

Although *Galdieria* photosynthetic activity supported greater *Methylacidiphilum* biomass in coculture (Table 5.2), and O_2 inlet affected CH_4 consumption rates (Figure 5.4d) in both axenic *Methylacidiphilum* and coculture chemostats (*p*-value < 0.001), there was no significant difference in CH_4 consumption rates between axenic *Methylacidiphilum* and coculture chemostats (*p*-value > 0.05). For the axenic *Methylacidiphilum* sp. RTK17.1 chemostat, CH_4 removal was 37.9 (± 1.4) % at 1.0 % O_2 inlet, and 62.9 (± 1.4) % at 3.0 % O_2 inlet. For the coculture the removal was 37.5 (± 1.0) % and 63.4 (± 1.1) % respectively. Thus, the coculture displayed a slower specific CH_4 consumption rate than the axenic methanotroph (Figure

5.4e). A similar observation was recently reported by Chidambarampadmavathy, *et al*. (2017) for cultures of mixed methanotrophic community structures enriched from landfill and compost soils. For the first 5 days of culture, when O_2 was in excess, methane removal and methane oxidation rates were correlated to biomass increases. After the 5th day, CH_4 consumption was stable and independent of biomass concentrations. Another similar case was reported for cocultures of the methanotroph *Methylocystis bryophila* and the microalgae *Scenedesmus obliquus* by Li *et al.* (2022), at high O_2 concentrations the final metabolite for CH_4 tended to be CO_2, while at low O_2 concentrations it tended to be extracellular organic compounds. Additionally, at more dilute O_2 concentrations the yield of biomass on CH_4 for *M*. *bryophilia* was lesser. Each of these observations can be explained by Kalyuzhnaya *et al.* (2013), who reported that in *M*. *alcaliphilum* 20Z, at low O_2 concentrations, CH_4 utilization switched to a fermentation mode that led to excretion of formate, acetate, succinate, lactate, H_2, and hydroxybutyrate as end products, with little biomass synthesis. A transcriptomic study showed upregulation of the genes for mixed-acid fermentation and H_2 production pathways (Kalyuzhnaya *et al.*, 2013). A similar mechanism might be occurring in *Galdieria*-*Methylacidiphilum* cocultures: with the extra O_2 produced by *Galdieria* allowing CH_4 to be oxidized further and supply more energy for biomass generation. Transcriptome analysis of the axenic and coculture chemostats at different O_2 concentrations could help us ascertain if a similar mechanism does occur between *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK37.1. Even though CH_4 removal was not significantly different between chemostats, *Galdieria* presence was still beneficial, as the *Methylacidiphilum* in coculture displayed greater biomass yield on CH_4 than the axenic methanotroph (p-value < 0.0001) (Table 5.2).

Biomass yields for axenic *Methylacidiphilum* sp. RTK17.1 in chemostat were greater than what I previously reported (4.89 ± 0.17 g_{DW} mol_{CH4}^{-1}) (Section 2.3.2 , Table 2.2), however this was expected, as I previously used a 250 rpm agitation speed, which would more severely limit CH_4 due to mass transfer constraints. Yields were however consistent with reported values from Carere *et al.* (2017), 6.79 (± 0.55) g_{DW} mol_{CH4}^{-1} in chemostat culture (800 rpm, D = 0.48 day^{-1}, O_2 inlet concentration 3.5% v/v); and Carere *et al.* (2019), 6.29 (± 0.55) g_{DW} mol_{CH4}^{-1} (800 rpm, D = 0.48 day^{-1}, O_2 inlet concentration 3.5% (v/v), H_2 inlet concentration 0.4% v/v). In the coculture, total biomass yields were greater due to the inflationary contribution of *Galdieria* biomass, they were however lesser than what previously reported (13.0 ± 0.9 g_{DW} mol_{CH4}^{-1}) (Table 2.2). This is a consequence of the increased dilution rate used in this study, as it decreased concentration from 0.393 (± 0.014) g_{DW} L^{-1} at D = 0.167 day^{-1} to 0.253 (± 0.007) g_{DW} L^{-1} at D = 0.278 day^{-1}. This increase in biomass yield did not affect protein quality, as the obtained indispensable amino acid contents (Figure 5.5) in the chemostats were similar to the values previously reported (Figure 2.6). Furthermore, inlet O_2 concentrations did not greatly affect the amino acid distribution for each type of biomass (i.e. *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1, or coculture). This is consistent with reports for other microalgae and methanotrophs: biomass growth conditions largely affect macromolecular composition, but not the amino acid profile (Batista *et al.*, 2013; Overland *et al.*, 2010; Templeton & Laurens, 2015).

Chemostat coculture allowed me to better understand how O_2 concentration affects *Methylacidiphilum*-*Galdieria* interactions. I previously reported (Section 4.3.1) that batch cocultures without O_2 supplementation using initial *Methylacidphilum*:*Galdieria* mass ratios ≥ 0.23 (g_{DW} L^{-1}: g_{DW} L^{-1}) resulted in

coculture collapse. In chemostat coculture at inlet O_2 < 2.1% (v/v), the interaction was inhibitory for the microalga: *Methylacidiphilum* sp. RTK17.1 benefited from *Galdieria* sp. RTK.37.1, while at the same time harming the microalga by inducing chlorosis. At inlet O_2 concentrations > 2.1% (v/v) the relationship became neutral for the microalga, *Methylacidiphilum* displayed faster growth, presumably due to O_2 availability allowing CH_4 to be completely oxidized, without seemingly affecting *Galdieria*. I also observed this behaviour for batch cocultures with no O_2 supplementation and *Methylacidiphilum*:*Galdieria* mass ratios < 0.23 (Section 4.3.1). In batch cocultures when O_2 was replenished each day, there was no evidence of positive or negative interactions between *Methylacidiphilum* and *Galdieria*. Thus, O_2 concentration and net uptake/production rates determine the type of *Methylacidiphilum-Galdieria* interaction in cocultures.

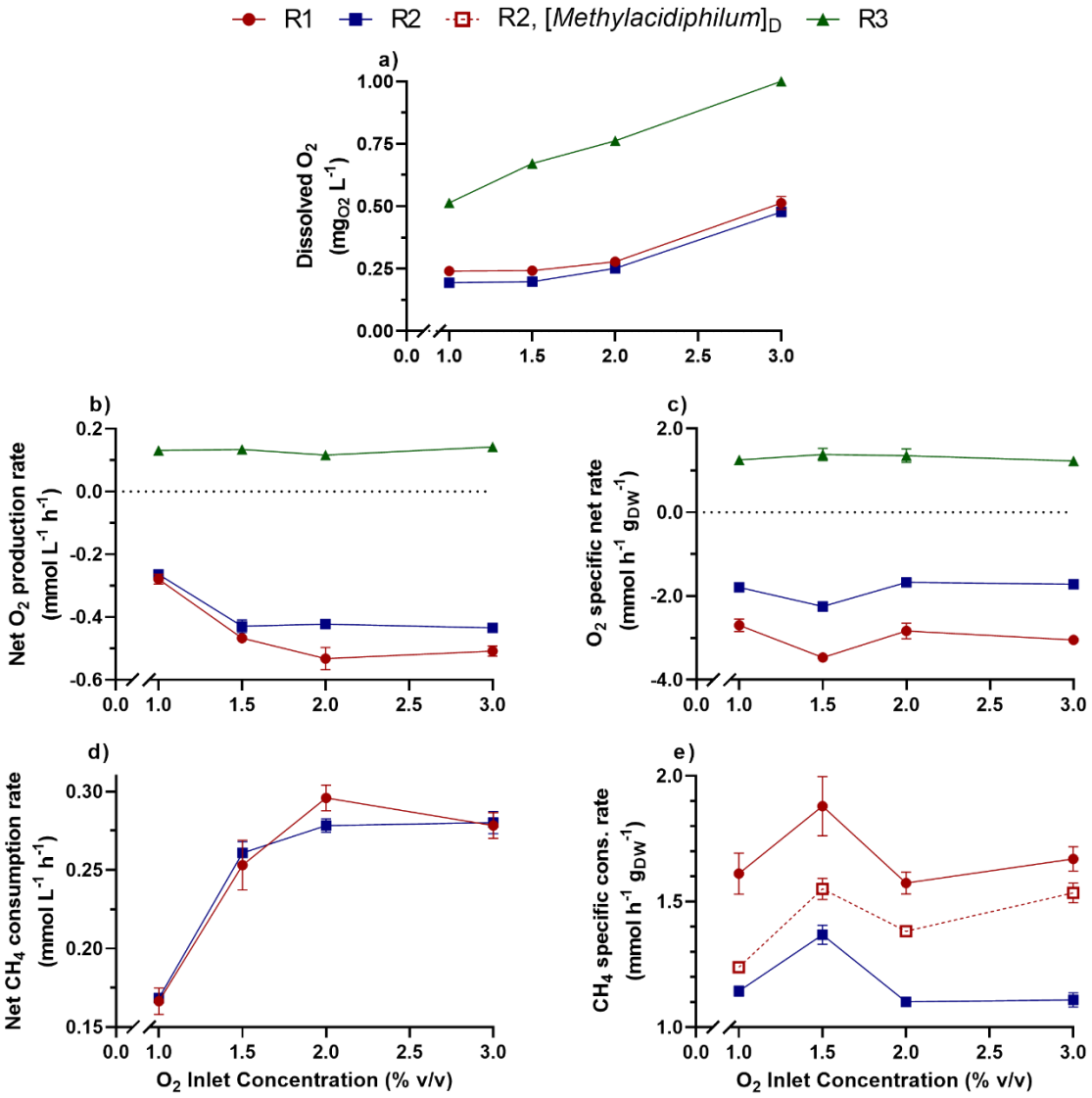


Figure 5.4. Steady state dissolved O_2 and production/consumption rates for *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 chemostat cocultures and axenic controls. a) Dissolved oxygen (D.O), b) net O_2 production rate, c) specific O_2 production rate, d) CH_4 consumption rate, and e) specific CH_4 consumption rate. All cultures were grown in 1 L stirred tank reactors at 45 °C, pH 2.5, 400 rpm, D = 0.278 day^{-1}, 900 mL working volume and 100 µmol m^{-2} s^{-1} measured at the middle of the empty reactor. Feed gas was supplied continuously at a 16 mL min^{-1} rate. All values are steady state averages of 5 consecutive measurements (n=5). Error bars represent one standard deviation.

Table 5.2. Biomass yields on methane for axenic *Methylacidiphilum* and coculture chemostat reactors.

Nominal Inlet O_2 Concentration	Biomass Yield (g_{DW} mol_{CH4}^{-1})[a]		
	Methylacidiphilum **(R1)[b]**	**[*Methylacidiphilum*]$_D$ (R2)[c]**	**Coculture (R2)[d]**
1.0	6.48 (± 0.33)	8.41 (± 0.15)	9.11 (± 0.16)
1.6	5.56 (± 0.34)	6.72 (± 0.18)	7.62 (± 0.20)
2.1	6.62 (± 0.18)	7.54 (± 0.11)	9.46 (± 0.14)
3.0	6.24 (± 0.18)	6.79 (± 0.17)	9.77 (± 0.25)

[a]All cultures were grown in 1 L stirred tank reactors at 45 °C, pH 2.5, 400 rpm, D = 0.278 day^{-1}, 900 mL working volume and 100 µmol m^{-2} s^{-1} measured at the middle of the empty reactor. Feed gas was supplied continuously at a 16 mL min^{-1} rate. All values are steady state averages of 5 consecutive measurements, numbers in parenthesis represent standard deviation (n=5).
[b]For *Methylacidiphilum* sp. RTK17.1 in axenic chemostat.
[c]Calculated for only *Methylacidiphilum* sp. RTK17.1 biomass in coculture, using total CH_4 consumption and derived methanotroph concentrations using DSOF method.
[d]Calculated using total CH_4 consumption biomass of both *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1.

5.3.1 High Biomass Density Fed-Batch Coculture

For industrial production of single cell protein (SCP), concentrated biomass cultures are desirable, as these facilitate biomass recovery and decrease overall costs. I have established from batch (Section 4.3) and chemostat cocultures (this study) that O_2 limitation can severely affect growth of both *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK17.1 in cocultures. In dense cocultures, *Methylacidiphilum* growth could pose a challenge, as it could overwhelm *Galdieria* and consume O_2 at such a rate to severely limit it. I reported that for batch cocultures with no added O_2, at initial *Galdieria:Methylacidiphilum* mass ratios ≥ 0.23 growth was rapidly arrested due to chlorosis of *Galdieria*. Also in chemostat coculture, chlorosis (and consequently lower biomass yields) was detected at D.O. ≤ 0.198 (± 0.003) mg_{O2} L^{-1}. Thus, to obtain more concentrated cocultures, coculture performance (e.g. growth rates, DO content, gas consumption/production rates) should be monitored and bioreactor parameters should be dynamically adapted as required. Therefore, to approximate a semi-industrial bioprocess, a static liquid fed-batch coculture was performed under a continuous gas feeding regime. In this experiment, to avoid N or O_2 limitation I added ammonium chloride (NH_4Cl) when coculture O.D. surpassed 3.0, 6.0 and 9.0 A.U., and increased light intensity when *Galdieria* OD stagnated. I intended to increase feed gas flowrate or O_2 concentration if D.O. decreased ≤ 0.198 mg_{O2} L^{-1}, but this was never required. A summary of the changes made to the coculture reactor is provided in Table 5.3.

Table 5.3. Chronology of dynamic parameter changes made to high biomass density batch coculture of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in 1 L bioreactor.

Day	Change
1	Coculture chemostat (R2) switched to 'batch mode', inlet gas flowrate 16 mL min^{-1} (2.1% O_2, 1.0% CH_4, 75% CO_2), 400 rpm agitation.
11	Added 10 mL NH_4Cl (40 g L^{-1})
18	Increased light intensity to 150 µmol photons m^{-2} s^{-1}
24	Added 10 mL NH_4Cl (40 g L^{-1})
32	Increased light intensity to 200 µmol photons m^{-2} s^{-1}
35	Added 10 mL NH_4Cl (40 g L^{-1})

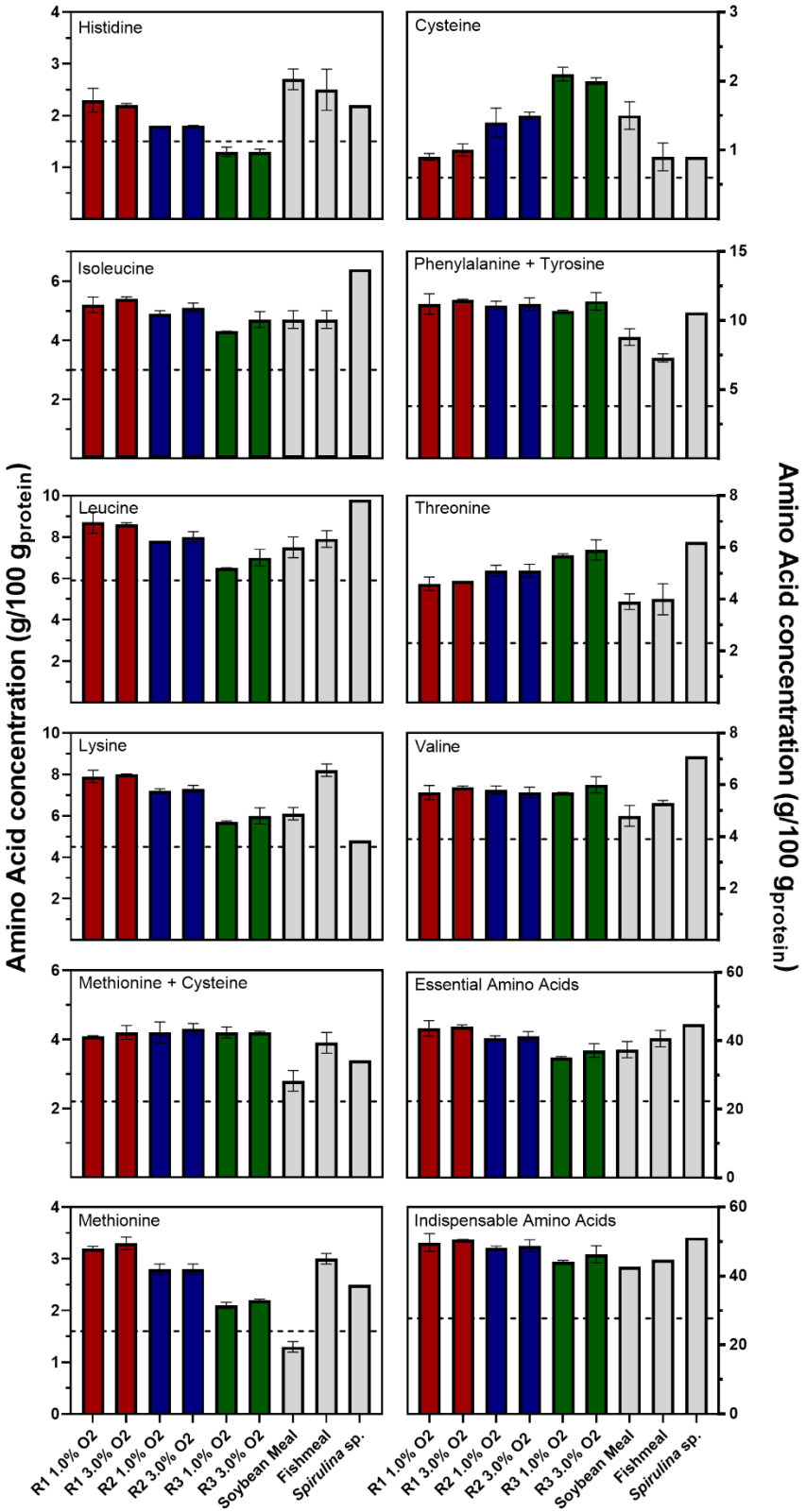


Figure 5.5. Indispensable amino acid composition comparison between chemostat cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1, and reference proteins. Error bars represent standard deviations with n = 2 technical replicates. Bars are colour coded red for *Methylacidiphilum* sp. RTK17.1, green for *Galdieria* sp. RTK37.1, blue for cocultures, and grey for reference proteins. The dashed black line represents the FAO/WHO/UNU reference protein. Reference proteins values soybean meal and fishmeal taken from Overland *et al* (2010), and *Spirulina* from Becker (2007).

The coculture took 42 days to reach stationary phase (Figure 5.6a), with a maximum biomass concentration of 2.73 g_{DW} L^{-1} achieved on day 38 (Figure 5.6b). During the first 11 days of culture, *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 concentrations increased linearly at similar rates (Figure 5.7b and Figure 5.7c). As the culture progressed, growth rates for both microorganisms slowed, with *Galdieria* growth rate decreasing down more than *Methylacidiphilum* (70 % and 39 % decrease in μ respectively by day 11). A similar behaviour was reported for batch cocultures of the methanotroph *Methylomicrobium buryatense* 5GB1 and the cyanobacteria *Arthrospira platensis* (Badr *et al.*, 2022). The authors attributed the decreased growth rate to light attenuation due to cell shading. Less available light slowed *A*. *platensis*, which in turn provided a diminishing O_2 supply to *M*. *buryatense*, thus limiting CH_4 oxidation and methanotroph growth (Badr *et al.*, 2022). Cell shading was the probable cause for *Galdieria* decreasing growth in our coculture, as I have previously observed that an OD_{600} = 0.44 A.U. of *Methylacidiphilum* sp. RTK17.1. slows down *Galdieria* growth by 11% (Section 4.3.1). For this study, light attenuation does not satisfactorily explain *Methylacidiphilum's* slowing growth rate (Figure 5.7b), as if this was the case, I would expect *Methylacidiphilum* and *Galdieria* growth rates to be proportional (as O_2 would become more limiting as *Galdieria* growth slowed down). In CH_4-replete cultures, volumetric CH_4 consumption rates are expected to increase proportionally to methanotroph growth to support the additional biomass. However, CH_4 consumption rates (Figure 5.7e) were constant for the duration of the experiment, which suggests a CH_4 mass transfer limitation. Consequently, *Methylacidiphilum* biomass concentrations increased linearly, and the specific growth rate slowed. Observed *Methylacidiphilum* sp. RTK17.1 growth rates were slower than the $\mu = 0.36\ day^{-1}$ reported by Carere *et al.* (2021). These slower growth rates are further evidence of CH_4 mass transfer limitation, as faster agitation rates (800 rpm) and a higher CH_4 concentration (10% v/v) were used in the Carere *et al.* (2021) study. In contrast, *Galdieria* sp. RTK37.1 growth rates ($\sim\mu = 0.10\ day^{-1}$) were consistent with reported rates for *Galdieria sulphuraria* 074G during photoautotrophic growth (Sloth *et al.*, 2006). As a consequence of *Galdieria* slower growth when compared to *Methylacidiphilum*, the relative microalgae concentration diminished over time. Badr *et al.* (2022) have recently reported that biomass ratios converge to approximately 3.5:1 (*Arthrospira*:*Methylomicrobium*) in coculture experiments, independent from the initial ratio used. It is possible that our *Methylacidiphilum*-*Galdieria* coculture could also converge to a constant ratio, but further experimentation would be required to test this hypothesis.

Dissolved oxygen values (Figure 5.7d) did not decrease sufficiently to induce coproporphyrin excretion (D.O. ≤ 0.198 ± 0.003 mg_{O2} L^{-1}). As methanotroph growth was constrained by CH_4 limitation, it was unable to overwhelm *Galdieria* and cause O_2 limitation. Thus, net O_2 consumption rates were stable (Figure 5.7e). On day 32 *Galdieria* reached stationary phase and pigments started to degrade (Figure 5.7f). Increasing light intensity did not enhance microalgae growth rate. As both ammonium and O_2 were available, and no coproporphyrin excretion was detected, it is possible that another nutrient was depleted from the media. Sloth, Jensen, Pleissner, and Eriksen (2017) reported that *Galdieria sulphuraria* grown heterotrophically on restaurant and bakery waste increased phycocyanin production 2.5 to 5-fold when supplemented with unspecified inorganic nutrients (not ammonium). An alternative explanation would be photoinhibition. Thangaraj *et al.* (2011) reported that laboratory cultures of *Galdieria* spp. can be inhibited by light intensities > 40 μmol photons m^{-2} s^{-1} as its photosynthetic systems are adapted to the low light conditions typical of endolithic habitats where this microalga thrives. Sloth *et al.* (2006) reported slowing growth rates for *Galdieria sulphuraria* at light intensities > 198 μmol photons m^{-2} s^{-1}, and Oesterhelt *et al.* (2007) found

strong photoinhibition at > 225 µmol photons m^{-2} s^{-1}. Photoinhibition is also consistent with the decrease in pigment content, as microalgae exposed to high light intensities supress chlorophyll production and synthesise carotenoids to reduce the negative effects of excess light (Wágner, Valverde-Pérez, & Plósz, 2018). Additionally, light intensities > 100 µmol photons m^{-2} s^{-1} decreased yield of phycocyanin in continuous culture of mixotrophic *Galdieria sulphuraria* 074G (Sloth *et al.*, 2006). As a consequence of *Galdieria* growth stopping and of pigment degradation following day 35, coculture O_2 consumption rates notably increased until day 42 when *Methylacidiphilum* reached stationary phase.

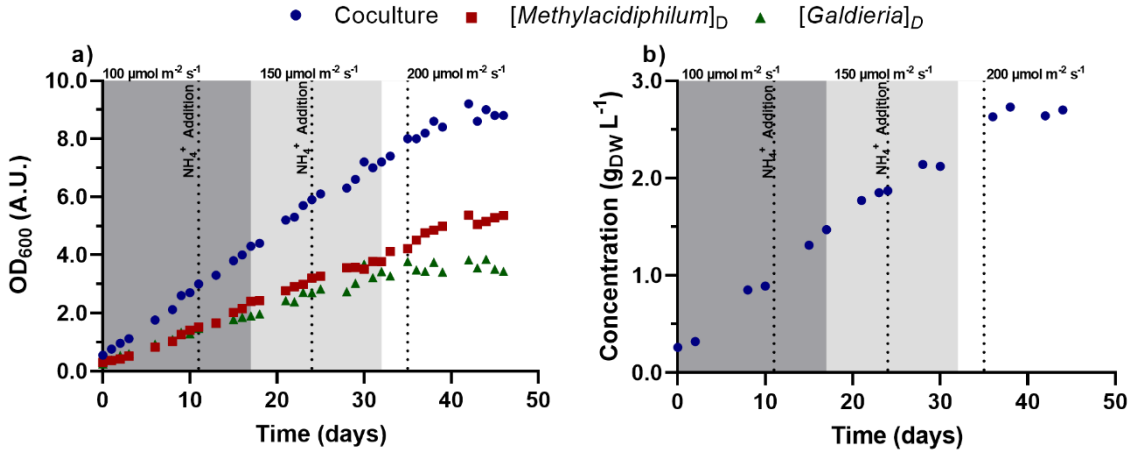


Figure 5.6. Batch coculture concentration profile for *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1. a) OD_{600} of coculture and relative abundance of *Methylacidiphilum* and *Galdieria*. Methanotroph and microalgae abundance in cocultures were calculated using the DSOF method. b) Total biomass concentration measured gravimetrically. Dashed vertical lines represent addition 10 mL of ammonium chloride (40 g L^{-1}) to the reactor. Shading indicates the light intensity used: 100 µmol photons m^{-2} s^{-1} (dark grey), 150 µmol photons m^{-2} s^{-1} (light grey), and 200 µmol photons m^{-2} s^{-1} (white). Coculture grown in a 1 L stirred tank reactor at 45 °C, pH 2.5, 400 rpm. Feed gas was supplied continuously at a 16 mL min^{-1} rate.

The maximum coculture biomass concentration achieved (2.73 g_{DW} L^{-1}) is comparable to other photoautotroph-methanotroph batch cocultures, albeit at a much slower rate. Rasouli *et al.* (2018) reported a biomass concentration of 1.49 g_{DW} L^{-1} after 20 h for cocultures of *M. capsulatus* and C. *sorokiniana* (maximum growth rate of 0.21 day^{-1}). Badr *et al.* (2022) reported a biomass concentration of 2.0 g_{DW} L^{-1} in a 100-h batch coculture of *M. buryatense* 5GB1 and *A. platensis*. Finally, Li *et al.* (2022) reported a biomass concentration of 0.968 g_{DW} L^{-1} following 200 h incubation of a batch coculture consisting of the methanotroph *Methylocystis bryophila* and the microalga *Scenedesmus obliquus*. Nevertheless, several strategies could be used to improve growth rates for the *Methylacidiphilum-Galdieria* coculture. Firstly, CH_4 mass transfer can be increased. For methanotroph reactors increasing methane mass transfer has a major effect on biomass concentration, yields, and growth rates (Kerckhof *et al.*, 2021). Increasing the gas transfer coefficient in U-Loop reactors by a factor of 2.5 has been shown to increase methanotroph concentration 3-fold (Kerckhof *et al.*, 2021). Increasing agitation speed would also improve mass transfer, in a *Methylacidiphilum* sp. RTK17.1 chemostat decreasing agitation from 800 to 250 rpm decreased OD_{600} from 0.92 to 0.29, and CH_4 removal from 87.2 to 40.5% (Section 2.3.2). However, significantly increasing agitation speed could harm the larger algae cells. The maximum reported agitation rate for *Galdieria* spp. in a stirred tank reactor is 500 rpm (Graverholt & Eriksen, 2007). Increasing pressure would also improve gas mass transfer into the liquid phase. I have previously increased the average growth rate of *Methylacidiphilum* sp. RTK17.1 in a 10 L STR reactor by 766% (from 0.094 to 0.720 day^{-1}) by increasing

gauge pressure from 0 to 500 mbar (Table H.1). Increasing *Methylacidiphilum* growth rate in this way would, however, also likely increase O_2 uptake rates, cell shading and probably the *Methylacidiphilum:Galdieria ratio.* Therefore D.O. could decrease below 0.198 mg O_2 L^{-1} and trigger chlorosis in *Galdieria*. To avoid this sequential cultivation of *Galdieria* and *Methylacidiphilum* could be helpful. In such a system, axenic *Galdieria* sp. RTK37.1 could be cultured to enrich O_2 content of a gas stream, which would then be fed to a *Methylacidiphilum* reactor. By separating both systems, optimum culture conditions could be used for each microorganism and O_2 limitation induced chlorosis could be avoided in *Galdieria*. However further research would be required to ascertain if better yields and titres could be obtained in a sequential culture vs a batch coculture.

5.4 Conclusions

Chemostat coculturing has resolved our understanding of how O_2 concentration affects *Methylacidiphilum-Galdieria* interactions. O_2 concentration and net uptake/production rates determine the nature of *Methylacidiphilum-Galdieria* interaction in cocultures. In chemostat coculture at inlet O_2 < 2.1% (D.O ≤ 0.198 ± 0.003 mg_{O2} L^{-1}), the interaction was inhibitory for the microalga: *Methylacidiphilum* sp. RTK17.1 benefited from *Galdieria* sp. RTK.37.1, while at the same time harming the microalga by inducing chlorosis. At inlet O_2 concentrations > 2.1% (v/v) the relationship became neutral for the microalga, *Methylacidiphilum* had faster growth, presumably due to O_2 availability allowing CH_4 to be oxidized further, without seemingly affecting *Galdieria*. Transcriptome analysis of the steady state axenic and coculture chemostats at the least and greatest O_2 inlet concentrations would help clarify the mechanism that drives the change in relationship between *Galdieria* sp. RTK37.1 and *Methylacidiphilum* sp. RTK37.1. Findings from these experiments were then used to continuously modify conditions within a high biomass density fed-batch coculture in such a way to obtain a more concentrated *Methylacidiphilum-Galdieria* coculture. I obtained a biomass concentration of 2.73 g_{DW} L^{-1} after 38 days of culture, the most concentrated *Methylacidiphilum-Galdieria* coculture reported to date. Further increases in coculture performance (growth rates, yields, gas consumption/production rates) will require increasing CH_4 mass transfer to the culture without triggering O_2 limited chlorosis. The aquired knowledge of the nature of *Methylacidiphilum-Galdieria* interactions is expected to assist with conceptualization and design of larger scale methanotroph-photoautotroph bioprocesses.

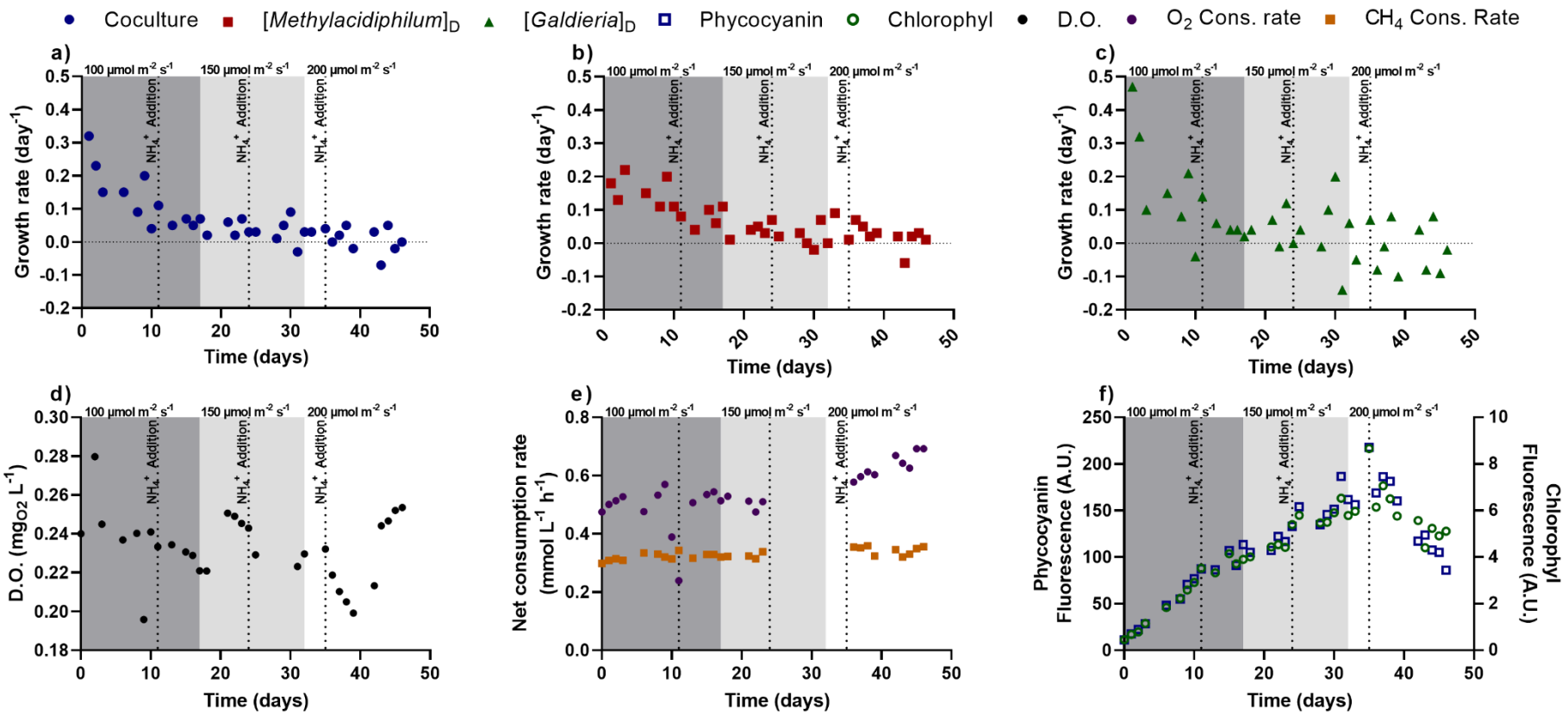


Figure 5.7. Batch coculture of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1: a) Coculture specific growth rate, b) *Methylacidiphilum* sp. RTK17.1 specific growth rate, c) *Galdieria* sp. RTK37.1 specific growth rate d) Dissolved O_2. growth rate, e), Net CO_2 and CH_4 consumption rates, and f) Phycocyanin and chlorophyll content. Dashed vertical lines represent addition 10 mL of ammonium chloride (40 g L^{-1}) to the reactor. Shading indicates the light intensity used: 100 µmol photons m^{-2} s^{-1} (dark grey), 150 µmol photons m^{-2} s^{-1} (light grey), and 200 µmol photons m^{-2} s^{-1}(white). Coculture grown in a 1 L stirred tank reactor at 45 °C, pH 2.5, 400 rpm. Feed gas was supplied continuously at a 16 mL min^{-1} rate.

Chapter 6. Summary, Future Research, and Conclusions.

6.1 Summary of Results

The aim of this project was to demonstrate proof-of-concept for a novel biotechnological platform with the capability to convert low value CH_4/CO_2 gas waste streams into supplementary biofeedstocks, by using a microbial extremophilic consortium composed of both methane oxidizers, and oxygenic photosynthetic CO_2 assimilating microorganisms. The first objective of this research was to determine if stable batch and steady state continuous cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 were possible. In Chapter 2 I found that stable coculture was achievable in both batch and chemostat configurations. In batch, *Galdieria* enhanced growth (29 %) and methane oxidation rates (300 %) of *Methylacidiphilum* significantly (*p*-values < 0.05 and < 0.001 respectively), and complete methane removal was achieved without formation of an explosive gas mixture (as defined by Janès *et al*, 2011). I found no evidence of microalgae inhibition by the methanotroph presence. In chemostat coculture experiments, *Galdieria* decreased net volumetric O_2 consumption by 46% in coculture, but its oxygenic activity was unable to supply *Methylacidiphilum* with the O_2 required for complete CH_4 removal. *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1, and their coculture each displayed similar nutritional profiles, with protein quality comparable to soybean meal and fishmeal feeds used for animals. The biomass needed to meet the daily indispensable amino acid requirements of a 62 kg human was found to be: 568 g for *Methylacidiphilum*, 804.8 g for *Galdieria*, and 753.7 g for the coculture, with histidine being the most limiting amino acid.

Existing photoautotroph-methanotroph coculture studies have suffered from a lack of rapid methods to quantitatively evaluate coculture dynamics. Therefore, the second objective of this research was to develop and validate a novel method to quickly measure the relative concentrations of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1. In Chapter 3 I developed and validated the DSOF method, a method which uses differences in settling velocities and autofluorescence of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 to determine the relative concentration of each coculture member. The validation of the method using artificial mixtures of *Methylacidiphilum* and *Galdieria* showed the absolute error of the derived biomass concentration values was negligible (≤ ±0.1 A.U.) when [*Galdieria*] ≤ 2.0 A.U. and [*Methylacidiphilum*] ≤ 1.5 A.U. These errors increase to ± 0.2 A.U, for 2.0 A.U. < $[Galdieria]_K$ ≤ 3.23 A.U. Overall, the DSOF method tended to underestimate *Methylacidiphilum* concentrations, likely because the method assumes no methanotroph cells settle during centrifugation. DSOF validation in actively growing cocultures showed that the derived *Methylacidiphilum-Galdieria* concentrations were consistent with their expected growth behaviour and prior observations. In conclusion, the DSOF method was determined to be a simple, quick, and accurate method to quantify the relative concentration of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture.

The third objective of this research was to evaluate the influence of *Methylacidiphilum*:*Galdieria* mass ratios on coculture performance. In addition, the role of O_2 concentration in *Methylacidiphilum-Galdieria* interactions in batch cultures was investigated. In Chapter 4 I found in serum bottle cocultures with variable initial *Methylacidiphilum*:*Galdieria* mass ratios (with and without CH_4), that at mass ratios < 0.23, cocultures with CH_4 fixed more net carbon, as the microalgae supplied the O_2 required for methane oxidation. However, CH_4-containing cocultures with initial *Methylacidiphilum*:*Galdieria* mass ratios ≥ 0.23 (g_{DW} L^{-1}: g_{DW} L^{-1}) collapsed: growth rate and net carbon fixation decreased significantly, coproporphyrin was excreted, and *Galdieria* suffered chlorosis. Coproporphyrin excretion and subsequent chlorosis was triggered by O_2 limitation, as neither was observed if O_2 and CH_4 were regularly replenished in cocultures. Finally, I showed that coproporphyrin excretion and chlorosis was reversible, as CH_4 removal from chlorotic cocultures resulted in coproporphyrin reabsorption and a resumption of pigment biosynthesis. Collectively, these results indicate that photoautotrophically grown *Galdieria* sp. RTK37.1 requires a minimum concentration of O_2 in the media to enable adequate pigment production, and that *Methylacidiphilum*'s high affinity for O_2 can trigger O_2 limitation in the microalga.

The final objective of this research was to study the interactions between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in dilute O_2 environments during steady state continuous coculture. I found that O_2 concentration and net uptake/production rates determine the nature of *Methylacidiphilum-Galdieria* interaction in cocultures. In chemostat coculture at inlet O_2 < 2.1% (D.O ≤ 0.198 ± 0.003 mg_{O2} L^{-1}), the interaction was inhibitory for the microalga: *Methylacidiphilum* sp. RTK17.1 benefited from *Galdieria* sp. RTK.37.1, while at the same time harming the microalga by inducing O_2-limitation chlorosis. At inlet O_2 between 2.1-3.0 % v/v the relationship became neutral for the microalga: *Methylacidiphilum* had faster growth, presumably due to O_2 availability allowing CH_4 to be oxidized further, without seemingly affecting *Galdieria*. Taking into account these results I was able to continuously modify conditions in a fed-batch coculture in such a way as to obtain a biomass concentration of 2.73 g_{DW} L^{-1} after 38 days of cultivation. This represents the most concentrated *Methylacidiphilum-Galdieria* coculture achieved to date and offers insight regarding the potential scalability of this bioprocess.

6.2 Future research

Methylacidiphilum sp. RTK17.1, *Galdieria* sp. RTK37.1 and coculture suitability as SCP

This research has revealed that the amino acid profiles of *Methylacidiphilum* sp. RTK17.1, *Galdieria* sp. RTK37.1 and their cocultures each exhibit a similar nutritional quality to soybean meal and fishmeal and could potentially be used as feed for animals. Their protein contents are also similar to other SCP sources. However further research is required to assess if *Galdieria* and *Methylacidiphilum* biomass is suitable as safe SCP source. Nucleic acid concentration for the 3 biomass types needs to be measured, as high concentrations could cause kidney stone formation and gout in humans (Zepka *et al.*, 2010). High nucleic acid concentration is a problem mainly in bacterial SCP (Anupama & Ravindra, 2000). If its concentration in *Methylacidiphilum* or coculture biomass is too high, then conditions favouring *Galdieria* growth (e.g. CH_4 limiting) could potentially diminish nucleic acid to acceptable concentrations.

Digestibility is a usual problem of microalgal SCP, as the cell wall cannot be degraded by most animals (Anupama & Ravindra, 2000). As other red algae lack rigid cell walls and thus are more digestible, I expect the same to hold true for *Galdieria*. Sinetova *et al.* (2006) reported that nitrogen starvation reduced cell wall thickness for *Galdieria sulphuraria*, so cultures under nitrogen depleted conditions could help address digestibility problems. A potentially undesirable consequence of this approach, however, is that nitrogen starvation also stimulates glycogen accumulation and thusly would decrease the protein concentration of both *Galdieria* and *Methylacidiphilum* biomass. In our research O_2-limited chlorosis behaved similarly to N_2-starvation chlorosis, so it is possible that limiting O_2 could also help reduce the cell wall thickness without influencing *Methylacidiphilum* protein concentration. Finally, both the toxicity and palatability of axenic cultures and cocultures needs to be studied.

Improvements to the DSOF method

While the DSOF method is quick, easy, and reliable, additional research is required to validate its broad utility to other methanotroph-photoautotroph cocultures. In Chapter 3 I highlight some of the research required for this. Firstly, the DSOF method requires validation under nitrogen limiting conditions. Nitrogen limitation leads to glycogen accumulation in both *Methylacidiphilum* and *Galdieria* (Graziani *et al.*, 2013; Sydney *et al.*, 2019), and chlorosis in *Galdieria* (Salbitani & Carfagna, 2020). Both effects are likely to considerably increase measurement error, so validation in N-depleted cultures would indicate whether the DSOF method remained accurate and precise under N-starvation. As an increase in microalgae cell size would also be expected with N-depletion (Sinetova *et al*., 2006) it could be possible to decrease centrifugation time and/or force to counteract such errors. This is also true for denser cocultures, where observed errors were greater. Decreasing either parameter is likely to diminish the amount of removed *Galdieria* cells after centrifugation, which could decrease the method resolution, so optimization of the method centrifugation time and force is required.

I validated the DSOF method with defined synthetic concentration mixtures and in live cocultures. For the live cocultures validation, I relied on cocultures where the growth of one of the partners was inhibited, and on the expected growth for a non-inhibited coculture. I could improve on this by comparing the DSOF method with other established methods with methanotroph-microalgae pairs where such methods have been shown to give satisfactory results, e.g., the EC method, proposed by Badr *et al.* (2020), with cocultures of *Methylococcus capsulatus* and *Chlorella sorokiniana*. Flow cytometry could also be used to compare the results of both methods (Badr *et al.*, 2020).

I observed that the DSOF method tended to underestimate *Methylacidiphilum* concentrations, likely because the method assumes no methanotroph cells are settled during centrifugation. In order to improve the method a concentration dependant correction factor could be added. The correction factor could be implemented by adding a *Methylacidiphilum* sedimentation term in Equation 3.2, then modelling said sedimentation using Stokes Law. Then model could then be calibrated with *Methylacidiphilum* and *Galdieria* mixtures of varying concentrations. Flow cytometry could be used to validate the results by measuring cell concentrations before and after centrifugation.

Coproporphyrin biological function in mixed cultures

In Chapter 4, results show *Methylacidiphilum* sp. RTK17.1 can cause O_2 limitation for *Galdieria* sp. RTK37.1 growing photoautotrophically, which triggers coproporphyrin excretion and chlorosis. Despite this novel finding, there are still several unanswered questions about the underlying physiology/biochemistry of this process. Coproporphyrin excretion has been reported for both mixotrophic (Zhu *et al.*, 2022) and heterotrophic (Sarian *et al.*, 2016) *Galdieria*, and I showed that it occurs in photoautotrophic cultures under O_2 limitation. However, as it was *Methylacidiphilum* O_2 consumption that triggered chlorosis, all photoautotrophic *Galdieria* coproporphyrin excretion was in coculture. To fully demonstrate that the cause is only O_2 concentration, I would need to trigger O_2-limiting chlorosis in axenic autotrophic *Galdieria*. This could not be done in batch culture, as produced O_2 would inevitably accumulate. I believe that continuously sparging O_2 free gas into either a static or continuous culture would have a similar effect to *Methylacidiphilum* O_2 consumption, as a sufficiently high flowrate would strip dissolved O_2. Such an experiment would also help in determining the threshold dissolved O_2 concentration below which coproporphyrin excretion is induced (by increasing gas flowrate, allowing the system to reach steady state, and then repeating until coproporphyrin is detectable). I had plans on performing such an experiment for this research but were not able due to reactor and time constraints.

It remains unknown whether coproporphyrin excretion facilitates any biological function (e.g. nutrient scavenging). Under N-starvation, Sinetova *et al.* (2006) showed that the structural changes in *Galdieria sulphuraria* cells served to maintain cell integrity, and through erythrosine staining found that the proportion of cell death was similar to N-replete grown cells. A similar mechanism might be at work with O_2 limitation. I found that cells that had undergone O_2-limitation induced chlorosis could recover, but the process took a relatively long time. I assumed this was because the resumption of pigment biosynthesis, starting from a position of depleted coproporphyrinogen III intermediate supply, was a slow process. However, the observed lag phase could also have been the result of cells dying as a consequence of chlorosis. Determining if *Galdieria* sp. RTK37.1 cells die or not after O_2-limiting chlorosis (via erythrosine staining or similar) could be the first step in determining the biological function of coproporphyrin excretion.

I also found that excreted coproporphyrin fluorescence decreased once *Methylacidiphilum* CH_4 oxidation was stopped and O_2 was allowed to accumulate in the media. As pigment synthesis followed, I speculated that *Galdieria* sp. RTK37.1 could reabsorb coproporphyrin and use the intermediate to synthesise pigments. To test this hypothesis further, several experiments focusing uptake by *Galdieria* could be helpful. First, if coproporphyrin was indeed being reabsorbed and used for pigment biosynthesis, I would expect that recovery from chlorosis would be slower if the coproporphyrin was removed from the media. To test this, we could coculture *Galdieria* and *Methylacidiphilum* in such a way to trigger chlorosis, then we could centrifuge the biomass and resuspend it in different media (e.g. fresh media without coproporphyrin, the spent media with coproporphyrin, and fresh media spiked with

different concentrations of coproporphyrin). Further controls of media containing coproporphyrin but no *Galdieria* cells, to rule out chemical degradation, should be added. If the cultures with coproporphyrin demonstrate recovering from chlorosis faster than those without, this would be further evidence of the reabsorption/synthesis hypothesis. Additionally, coproporphyrin spiked cultures of non O_2-limited *Galdieria* could be useful to ascertain if non chlorotic *Galdieria* could uptake coproporphyrin. Since in cocultures of different microorganisms coproporphyrin has been reported to work both as a growth promoter and inhibitor (Bhuiyan *et al.*, 2016; Cleary *et al.*, 2018), it is possible that its presence could similarly affect *Galdieria* growth.

Galdieria spp., frequently thrives in endolithic habitats, where low light intensities and O_2 concentrations would be expected (Thangaraj *et al.*, 2011). Under such conditions I would expect Galdieria to grow hetero- and mixotrophically and thus compete for O_2. Coproporphyrin is used by other microorganisms, like *Paracoccus denitrificans (Anttila et al., 2011)*, to scavenge nutrients. I speculate that coproporphyrin then could be used as a mechanism to control other microorganisms: when O_2 concentration decreases, coproporphyrin gets excreted, it scavenges some nutrient (like copper), competing microorganisms concentrations decrease, thus O_2 concentration then starts increasing and coproporphyrin gets reabsorbed. *Galdieria sulphuraria* has been used to recover dissolved copper and lanthanoids under low O_2 concentrations (Minoda *et al.*, 2015), so this hypothesis is reasonable. Monitoring lanthanoid and copper concentrations, before, during and after O_2 limiting chlorosis (with non-chlorotic controls) could help in assessing this possibility. If indeed *Galdieria* sp. RTK37.1 recovers lanthanoids via coproporphyrin excretion/reabsorption, this research could meaningfully improve *Galdieria*-based biosorption (e.g. growing *Galdieria* heterotrophically to high concentrations, then limiting O_2 to induce coproporphyrin excretion, allowing coproporphyrin to bond with the metals, and then increasing O_2 for the coproporphyrin-associated target metals to be reabsorbed). It is also possible that coproporphyrin could inhibit *Methylacidiphilum* sp. RTK17.1 growth. In Chapter 5 I observed no such inhibition, but *Galdieria* sp. RTK37.1 concentrations in the chemostat reactor were relatively low. To better understand the effects of coproporphyrin in *Methylacidiphilum*, I suggest steady state chemostat culture of the methanotroph with increasingly concentrated coproporphyrin supplemented media.

Transcriptome analysis in Methylacidiphilum sp. RTK17.1, *Galdieria* sp. RTK37.1 and coculture chemostats

In Chapter 5 I varied the O_2 inlet concentrations for axenic cultures and cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in steady state reactors, and I found the relationship between both microorganisms changed from synergistic to antagonistic as O_2 concentration decreased. Samples from the steady state reactors were taken and stored with RNA-later solution at -20 °C for a series of planned transcriptome experiments. Due to time constraints brought on by the COVID-19 pandemic, however, these analyses have not yet been performed. Transcriptome analysis of the steady state axenic and coculture chemostats, could provide evidence of whether *Galdieria* and *Methylacidiphilum* in coculture affect each other at the level of gene expression. This could help explain several of the

Galdieria-Methylacidiphilum interactions I observed. For example, in Chapter 5 I observed increased *Methylacidiphilum* growth in the coculture, when compared to the axenic chemostat, even though CH_4 oxidation was not affected. For M. *alcaliphilum* 20Z, Kalyuzhnaya *et al.* (2013) reported that at low O_2 concentrations, CH_4 utilization switched to a fermentation mode that led to production of several organic molecules and H_2, with little biomass production. Kalyuzhnaya *et al.* (2013) showed, through a transcriptomic study, upregulation of the genes for mixed-acid fermentation and H_2 production pathways. I speculated, that a similar mechanism was responsible for our findings, and the transcriptome analysis could support or disprove our hypothesis. Additionally, an upregulation of genes that encode for tetrapyrroles transporters for *Galdieria* under O_2 limitation, could support the hypothesis of coproporphyrin transportation/reabsorption.

Organic carbon cross feeding of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 in coculture

Both *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 have complex metabolisms and are capable of growing on a variety of different organic carbon substrates. Heterotrophic and mixotrophic growth has been confirmed on 27 different substrates for *Galdieria* spp. (Gross & Schnarrenberger, 1995; Vítová *et al.*, 2016), and *Methylacidiphilum* spp. growth has been confirmed on methanol (Dunfield *et al.*, 2007), formic acid (Carere *et al.*, 2021), ethane, and propane (Picone *et al.*, 2020). This metabolic flexibility suggests the possibility of growth using metabolites produced by the other species in coculture. Such cross feeding has been reported by Li *et al.* (2022) for cocultures of the microalgae *Scenedesmus obliquus* with the methanotroph *Methylocystis bryophila*, where organic carbon released by *M*. *bryophila* triggered a switch from autotrophic to mixotrophic growth in *S. obliquus*. To test if cross feeding could happen in *Methylacidiphilum-Galdieria* cocultures, heterotrophic growth of *Galdieria* should be attempted on *Methylacidiphilum* spent media (and vice-versa).

Modelling of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 coculture growth

I concluded on Chapter 5 that further increases in coculture performance (growth rates, yields, gas consumption/production rates) will require an increase in CH_4 mass transfer to the culture without triggering O_2 limited chlorosis. For this a working model that can describe *Methylacidiphilum-Galdieria* coculture would facilitate design of reaction systems. A simple semi-structured kinetic model similar to the one proposed by Badr *et al.* (2022) could be constructed and experimentally validated. Such a model uses Monod model to describe growth rates, yield coefficients for substrate consumption/product formation, and mass transfer coefficients, and Henry's Law to determine liquid concentrations from gas measurements. The required coefficients could be obtained from simple batch experiments. On first instance an O_2 limitation term could be introduced in the expression of *Galdieria* growth to account for O_2 limitation induced chlorosis. However, the relationship between the D.O. at which O_2 limitation starts and light intensities would need to be found. This could be done in chemostat culture by changing light intensity, allowing the system to reach steady state, and then increasing gas flowrate until

coproporphyrin is detected in the media. Such a model could then be validated under various conditions and used to predict the performance of new reaction systems or optimize existing ones.

Sequential axenic culture of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1

While cocultures of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 are interesting for valorisation of industrial waste gases, the yields and productivities I obtained in this research are much lesser when compared to other photoautotroph-methanotroph pairs (Section 5.3.1). Thus, it is important to investigate ways of improving coculture performance. One way to accomplish this could be decoupling *Galdieria* and *Methylacidiphilum*. *Methylacidiphilum* sp. RTK17.1 growth could be accelerated by increasing CH_4 mass transfer i.e., by faster agitation rates and increased pressure. However, the increased shear force, and/or oxygen uptake rate could potentially affect *Galdieria* productivity. Thus, separating each microorganism into their own vessel with interconnected gas streams, could allow separate optimization of their respective reactor conditions to increase growth rates and/or total yields. In such a system, axenic *Galdieria* sp. RTK37.1 could be cultured to enrich O_2 content of a gas stream, which would then be fed to a *Methylacidiphilum* reactor. Gas streams could be connected sequentially or some degree of gas recirculation between reactors could be done (to enhance mass transfer and cross feeding). However further research would be required to ascertain if better yields and titres could be obtained in a sequential culture vs. a coculture. Kinetic models, as I previously described, could facilitate, and complement this research. For example, optimization of parameters like gas recirculation flows, or volume of *Galdieria:Methylacidiphilum* reactor systems could be easily explored.

6.3 Conclusions

Methylacidiphilum sp. RTK17.1 and *Galdieria* sp. RTK37.1 can sustain stable cocultures in both batch and continuous configurations, and according to their amino acid profiles, *Methylacidiphilum*, *Galdieria*, and their cocultures exhibit a comparable nutritional quality to soybean meal and fishmeal and could potentially be used as feed for animals (H1).

Oxygen clearly plays a fundamental role in *Methylacidiphilum-Galdieria* interactions. In general, if O_2 is limiting *Methylacidiphilum* RTK 17.1 benefits from *Galdieria* photosynthetic activity. However, if *Methylacidiphilum* related O_2 consumption exceeds *Galdieria* photosynthetic O_2 production, the methanotroph can harm the microalga. This O_2 limitation in *Galdieria* causes the chlorophyll and phycocyanin intermediate, coproporphyrinogen III, to be excreted into the media and oxidized into coproporphyrin III. This stops pigment synthesis, which eventually results in pigment degradation (chlorosis). I showed that O_2-limiting chlorosis is reversible, however, as pigment synthesis eventually resumes if O_2 concentration is increased. In conclusion, balancing O_2 production/consumption is vital in *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 cocultures (H2).

O_2-limitation induced chlorosis is the reason why growth rates depend on the *Methilacidiphilum*:*Galdieria* mass ratio in batch cocultures without O_2 supplementation. When the *Methilacidiphilum*:*Galdieria* mass ratio is relatively low, the increased O_2 availability improves *Methylacidiphilum* CH_4 consumption and growth rates. However, when the ratio increases, the increased O_2 consumption triggers a vicious spiral: as coproporphyrin gets excreted, pigments degrade, then photosynthesis becomes impaired, no further O_2 can be produced which hinders pigment production further, and hence cocultures collapse. The exact ratio at which the synergistic interaction becomes antagonistic is probably dependent on light intensity and external supplies of O_2 and CH_4. This is evidenced by coproporphyrin excretion and chlorosis not occurring in coculture when O_2 is regularly replenished (H3).

The importance of balancing O_2 production/consumption is also evident in the synergistic/antagonistic change in chemostat coculture. At high O_2 concentrations I found no evidence of *Methylacidiphilum* and *Galdieria* affecting each other, however their relationship became first synergistic (*Methylacidiphilum* had faster growth, without seemingly affecting *Galdieria*), and later inhibitory (*Methylacidiphilum* sp. benefited from extra O_2 availability, while at the same time induced O_2-limitation chlorosis) as O_2 concentrations decreased. It is likely that further reducing O_2 would result in washout of both *Galdieria* and *Methylacidiphilum*. The nature of the interaction between *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 is dependent on the dissolved O_2 concentration, and thus, its monitoring and control is of vital importance for successful cocultures.

This research has made a significant contribution to the understanding of *Galdieria* spp., photoautotroph-methanotroph coculture in general, and more specifically to thermoacidophilic cocultures. Coproporphyrin excretion has been previously reported for *Galdieria* spp. (Sarian *et al.*, 2016; Zhu *et al.*, 2022), however this research is the first reported instance of it occurring during photoautotrophic growth, or of it being induced via the activity of another microorganism. This is a significant contribution to the understanding of coproporphyrin accumulation/excretion in *Galdieria* and provides further evidence that it is O_2 concentration and not C-source inhibition that causes the phenomena. Furthermore, coproporphyrin being reabsorbed by *Galdieria* spp. leads to new lines on inquiry regarding its biological function in mixed cultures. Regarding this, our research provides insight into how O_2 concentrations affect *Methylacidiphilum*-*Galdieria* cocultures. This interspecies dynamic will serve as the foundation for the conceptualization and design of systems to improve coculture performance. Additionally, developing of the DSOF method allows for the rapid and simple measuring of the relative concentration of microalgae and methanotrophs in coculture. This novel tool enables quasi-'real time' monitoring of coculture response to different growth conditions. Our research expands understanding of photoautotroph-methanotroph cocultures and provides a proof-of-principle for thermoacidophilic SCP bioprocesses. These thermoacidophilic cocultures, which have not previously been investigated, offer great potential to convert low (or negative) value industrial gas streams into valuable products (e.g. supplementary biofeedstocks).

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Appendix A. V4 Media Recipe

To make modified V4 media (Dunfield *et al.*, 2007), the nutrients specified in Table A.1 are dissolved into 1 L of deionized water, then the pH is adjusted to 2.5 using H_2SO_4 2 M, and finally the media is autoclaved.

Table A.1. Nutrients and micronutrients composition in 1 L of V4 media.

Nutrient	Amount
NH_4Cl	0.4 g
KH_2PO_4	0.05 g
$MgSO_4 \cdot 7H_2O$	0.02 g
$CaCl_2 \cdot 2H2O$	0.01 g
FeEDTA solution	3 mL
Trace element solution 1 for methanotrophs	3 mL
Trace metal solution for methanogens	1 mL
1mM Cerium sulfate solution	200 uL
1mM Lanthanum sulfate solution	200 uL

The FeEDTA solution is prepared by dissolving 1.54 g of $FeSO_4 \cdot 7H_2O$ and 2.06 g of Na_2EDTA in 1 L of deionized water. The Trace element solution for methanotrophs is prepared by dissolving the micronutrients specified in Table A.2 in 1 L of deionized water.

Table A.2. Mineral contents in 1 L of trace element solution 1 for methanotrophs.

Micronutrient	Amount
$ZnSO_4 \cdot 7\ H_2O$	0.44 g
$CuSO_4 \cdot 5\ H_2O$	0.20 g
MnCl 4H2O	0.19 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.06 g
H_3BO_3	0.10 g
$CoCl_2 \cdot 6\ H_2O$	0.08 g

To prepare the trace element solution, 1.5 g of Nitrilotriacetic acid is dissolved in 800 ml of deionized water and the pH is adjusted to 6.5 with KOH. Then the rest of minerals in Table A.3 are dissolved in order, the pH is then adjusted to 7.0, and the volume brought to 1 L with deionized water.

Table A.3. Mineral contents in 1 L of trace metal solution for methanogens.

Mineral	Amount
Nitrilotriacetic acid	1.5 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.2 g
Na_2SeO_3	0.2 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$MnSO_4 \cdot 2H_2O$	0.1 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
$Na_2WO_4 \cdot 2H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$AlCl_3 \cdot 6H_2O$	0.04 g
$NiCl_2 \cdot 6H_2O$	0.025 g
H_3BO_3	0.01 g
$CuSO_4 \cdot 5H_2O$	0.01 g

Appendix B. Optical density to concentration conversion factors

B.1. Galdieria sp. RTK37.1 OD_{600} to dry weight conversion factor

In order to obtain an OD_{600} to dry weight conversion factor for *Galdieria* sp. RTK37.1, 3 bottles of axenic microalgae were cultured as described in Section 2.2.2 . Biomass concentrations were determined gravimetrically as described in Section 4.2.5 . It was found that one unit of OD_{600} was equivalent to 0.308 g_{DW} L^{-1} for *Galdieria* sp. RTK37.1 (Figure B.1).

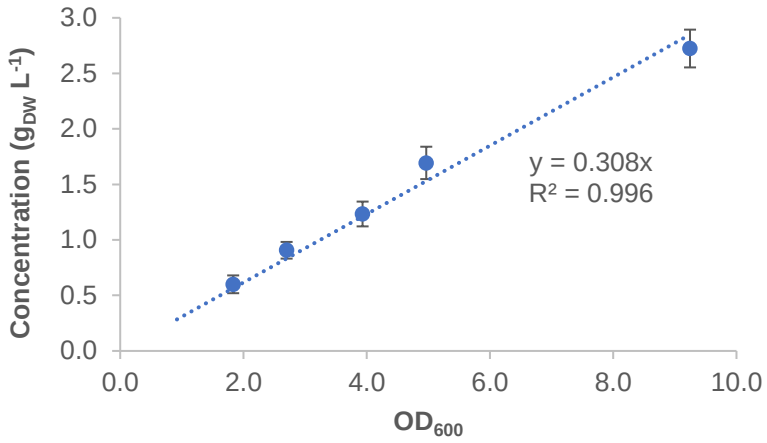

Figure B.1. Galdieria sp. RTK37.1 OD_{600} to dry weight conversion factor.

B.2. *Methylacidiphilum* sp. RTK17.1 OD_{600} to dry weight conversion factor

In order to obtain an OD_{600} to dry weight conversion factor for *Methylacidiphilum* sp. RTK17.1, axenic methanotroph was cultivated in batch, in a STR bioreactor as described in Section 2.2.1 . Biomass concentrations were determined gravimetrically as described in Section 4.2.5 . It was found that one unit of OD_{600} was equivalent to 0.435 g_{DW} L^{-1} for *Methylacidiphilum* sp. RTK17.1 (Figure B.2).

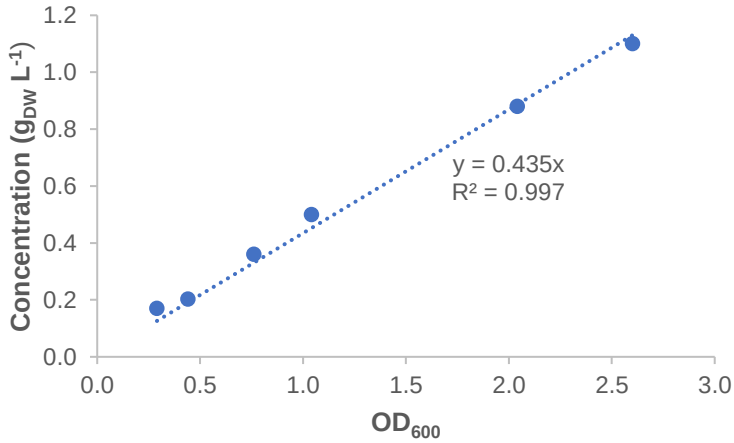

Figure B.2. *Methylacidiphilum* sp. RTK17.1 OD_{600} to dry weight conversion factor.

Appendix C: Preliminary experiments for DSOF method

C.1. OD_{600} values are additive for *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1

To test whether *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 OD_{600} values were additive, artificial "coculture" suspensions were prepared from axenic stock solutions across a range of predefined OD_{600} values and measured using a Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom). Measured suspension values were compared to "known" values (the summation of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 individual OD_{600} values).

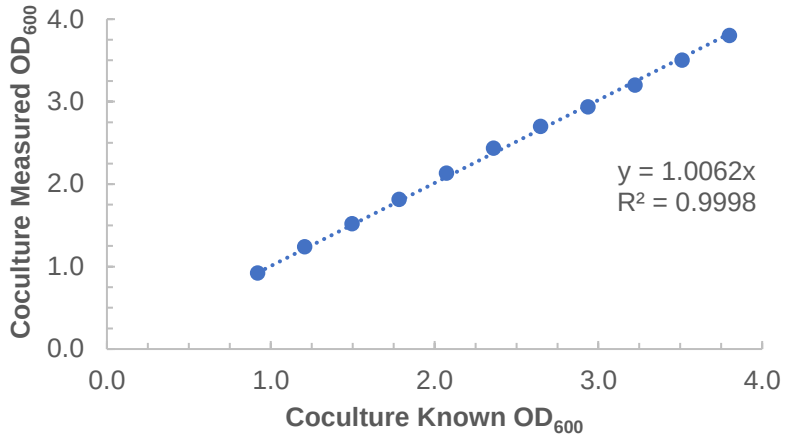

Figure C.1. Comparison of artificial "coculture suspension" OD_{600} with the summation of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 individual OD_{600} values.

C.2. There is no significant centrifugation of methanotrophic cells

To test whether methanotrophic cells were centrifugated to a relevant degree, axenic suspensions of *Methylacidiphilum* sp. RTK17.1 (OD_{600} = 0.88) and *Galdieria* sp. RTK37.1 (OD_{600} = 2.4) were centrifuged at 380 x g for variable periods of time a using microcentrifuge (Biofuge Pico, Heraeus Instruments). OD_{600} were measured from the suspensions and the supernatants using a Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom), and the fraction of supernatant retained biomass was then calculated. For times < 50 s, retained biomass for *Methylacidiphilum* was > 90%.

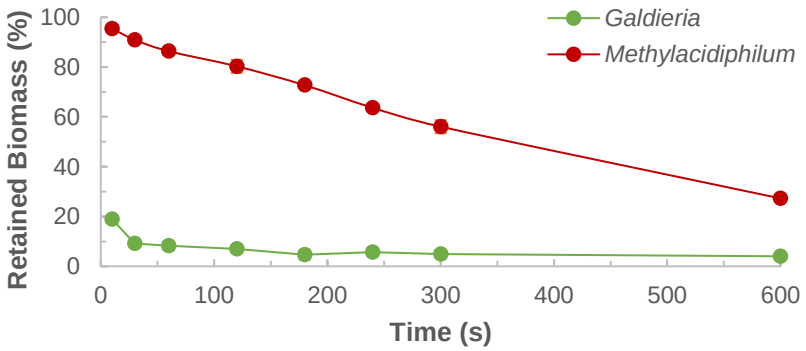

Figure C.2. Fraction of retained biomass in the supernatant for *Methylacidiphilum* sp. RTK17.1 (OD_{600} = 0.88) and *Galdieria* sp. RTK37.1 (OD_{600} = 2.4) suspensions.

C.3. The fraction of microalgae remaining in the supernatant can be approximated by the proportion of fluorescence of supernatant and original coculture

To test whether fluorescence (emission 670 nm, excitation at 590 nm) was proportional to OD_{600} for *Galdieria* sp. RTK37.1, and that *Methylacidiphilum* sp. RTK17.1 exhibited no fluorescence at 670 nm when excited at 590 nm, dilutions of stock solutions were made. The suspensions' OD_{600} were measured using an Ultrospec 10 cell density meter (Amersham Bioscience, United Kingdom), and the fluorescence (670 nm emission, 590 nm excitation) was measured using a microplate reader (Varioskan Lux,Thermo Scientific). *Galdieria* sp. RTK37.1 fluorescence was proportional to OD_{600}, and Methylacidiphilum sp. RTK17.1 showed no fluorescence.

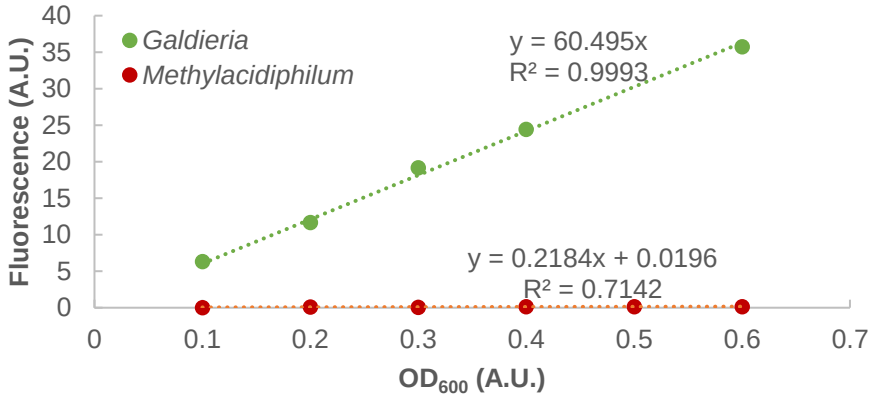

Figure C.3. Fluorescence (670 nm emission, 590 nm excitation) as a function of OD_{600} for suspensions of *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1.

To test whether, after centrifugation, supernatant *Galdieria* sp. RTK37.1 fluorescence (670 nm emission, 590 nm excitation) was still proportional to OD_{600}, G*aldieria* sp. RTK37.1 (OD_{600} = 2.4) suspensions were centrifuged at 380 x g for variable periods of time a using microcentrifuge (Biofuge Pico, Heraeus Instruments). Suspension and supernatant's OD_{600} and fluorescence were measured and the fraction of retained biomass and fluorescence were calculated. After centrifugation *Galdieria* sp. RTK37.1 fluorescence was proportional to OD_{600}.

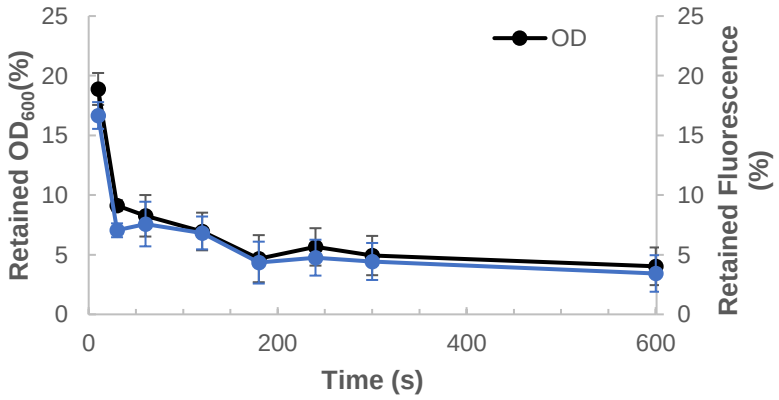

Figure C. 4. Fraction of retained OD_{600} and fluorescence (670 nm emission, 590 nm excitation) in the supernatant for suspensions of *Galdieria* sp. RTK37.1.

Appendix D: Variable Mass Ratio Coculture Experiments OD_{600} Temporal Profile

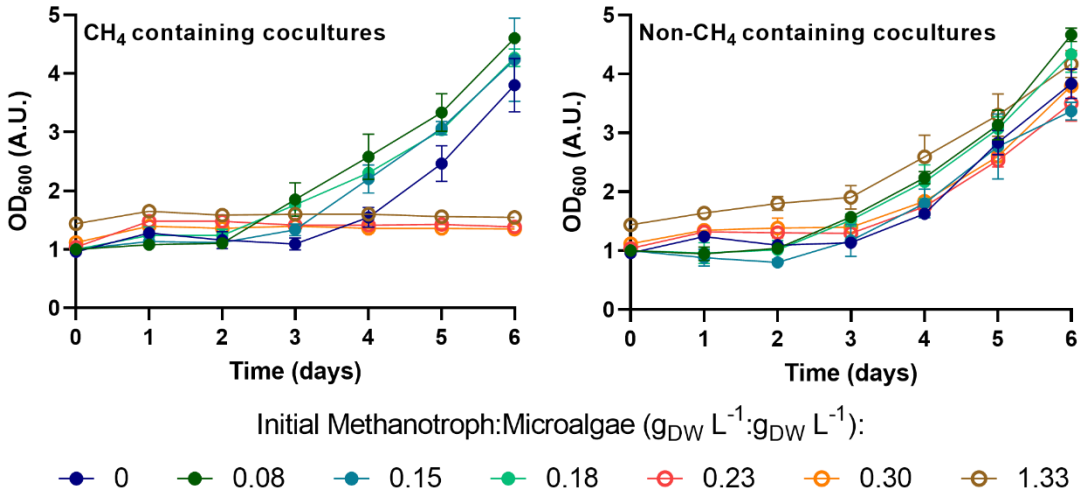

Figure D.1. Effect of the initial *Methylacidiphilum* RTK17.1 to *Galdieria* RTK37.1 concentration ratio on OD_{600} for cultures with and without CH_4 supplementation in the headspace.

Appendix E. LC-MS spectra of chlorotic coculture supernatant samples

The pooled supernatants of the chlorotic cocultures (Section 4.2.3) were analysed in an LC-MS as described in Section 4.2.4 . Results are shown in Figure E.1 and Figure E.2.

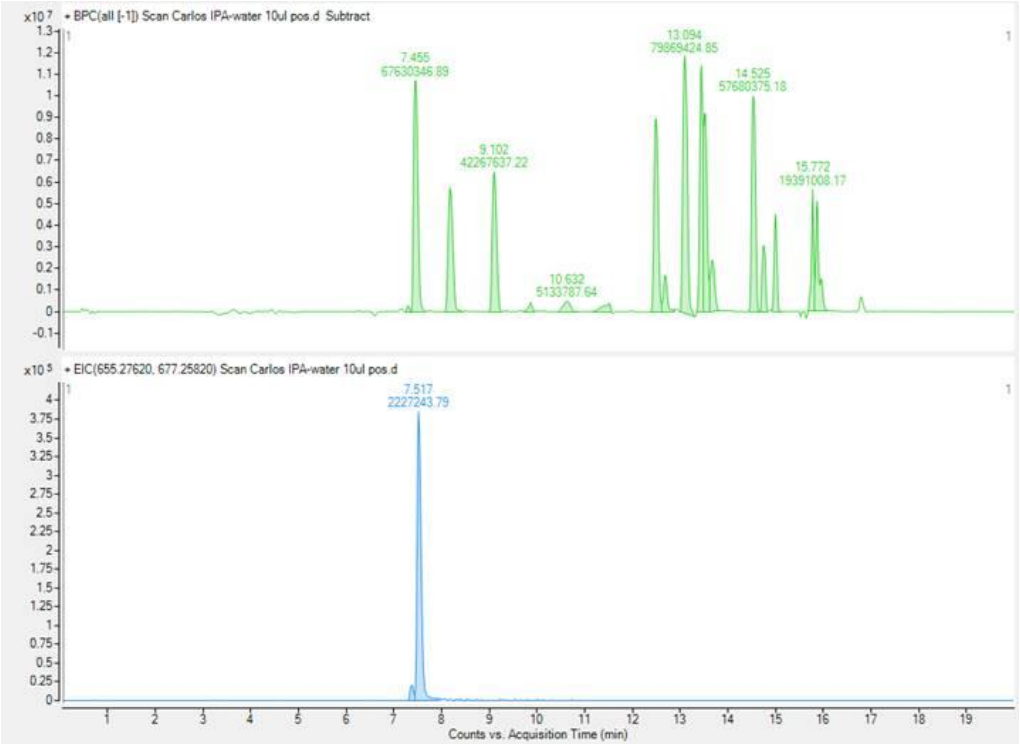

Figure E.1. BPC and EIC for the sample in IPA/water in positive ion mode.

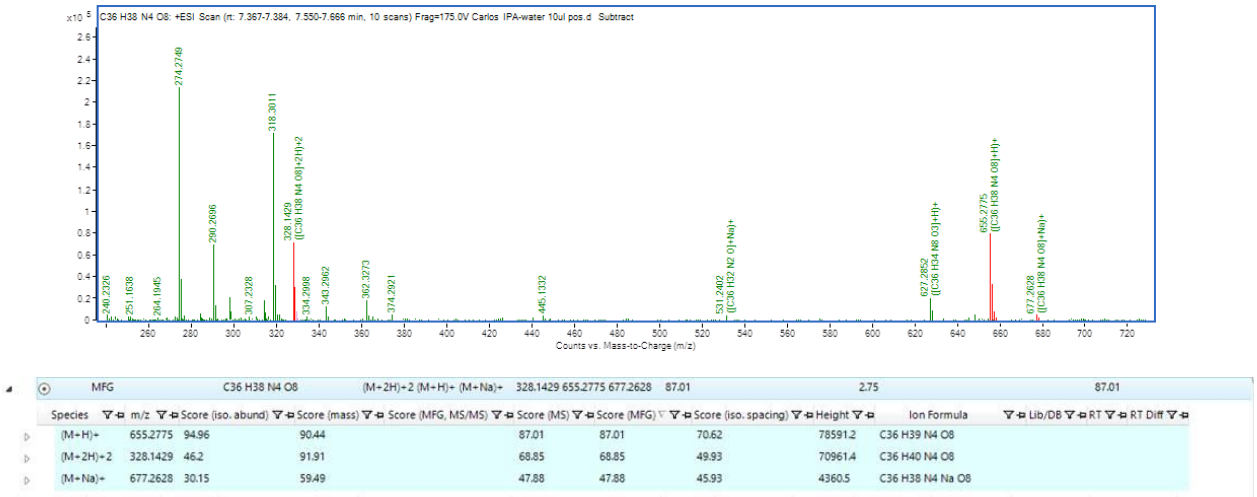

Figure E.2. Mass spectrum of the peak at r.t. 7.46 min showing the 677.2762 ($C_{36}H_{38}N_4O_8$, expected $[M+H]^+$) and the $[M+Na]^+$ and $[M+2H]^{2+}$ ions, and the formula matching scores.

In the sample the ion 677.2762 ($C_{36}H_{38}N_4O_8$, expected $[M+H]^+$ for coproporphyrin III) was observed in the peak at 7.46 min. This matches well with the calculated mass for this formula and in the mass spectrum for this peak there were also the masses for $[M+Na]^+$, and $[M+2H]^{2+}$ for $C_{36}H_{38}N_4O_8$. The results are consistent with coproporphyrin III presence in the sample.

Appendix F Multiple Comparisons Tests for Chemostat Reactors

Table F.1. Sidak's multiple comparisons tests between the means of *Methylacidiphilum* sp. RTK17.1 $OD6_{00}$ values at different inlet O_2 concentrations in the axenic (R1) chemostat.

Means Compared	*p*-values	Significant difference between means?
1.0 % vs 1.6 %	< 0.0001	Yes
1.0 % vs 2.1 %	< 0.0001	Yes
1.0 % vs 3.0 %	< 0.0001	Yes
1.6 % vs 2.1 %	< 0.0001	Yes
1.6 % vs 3.0 %	0.0037	Yes
2.1 % vs 3.0 %	0.0037	Yes

Table F.2. Sidak's multiple comparisons tests between the means of *Methylacidiphilum* sp. RTK17.1 $OD6_{00}$ values in axenic (R1) and coculture (R2) chemostats.

Inlet O_2 concentration (% v/v)	*p*-values	Significant difference between means?
1.0	< 0.0001	Yes
1.6	< 0.0001	Yes
2.1	0.0038	Yes
3.0	0.0007	Yes

Table F.3. Sidak's multiple comparisons tests between the means of *Methylacidiphilum* sp. RTK17.1 $OD6_{00}$ values at different inlet O_2 concentrations in the coculture (R2) chemostat.

Means Compared	*p*-values	Significant difference between means?
1.0 % vs 1.6 %	< 0.0001	Yes
1.0 % vs 2.1 %	< 0.0001	Yes
1.0 % vs 3.0 %	< 0.0001	Yes
1.6 % vs 2.1 %	0.9903	No
1.6 % vs 3.0 %	0.0895	No
2.1 % vs 3.0 %	0.0486	Yes

Table F.4. Sidak's multiple comparisons tests between the means of *Galdieria* sp. RTK37.1 OD_{600} values in axenic (R3) and coculture (R2) chemostats.

Inlet O_2 concentration (% v/v)	*p*-values	Significant difference between means?
1.0	< 0.0001	Yes
1.6	< 0.0001	Yes
2.1	< 0.0001	Yes
3.0	0.0172	Yes

Table F.5. Sidak's multiple comparisons tests between the means of *Galdieria* sp. RTK37.1 OD_{600} values at different inlet O_2 concentrations in the axenic (R3) chemostat.

Means Compared	*p*-values	Significant difference between means?
1.0 % vs 1.6 %	0.0127	Yes
1.0 % vs 2.1 %	0.0014	Yes
1.0 % vs 3.0 %	< 0.0001	Yes
1.6 % vs 2.1 %	< 0.0001	Yes
1.6 % vs 3.0 %	0.0034	Yes
2.1 % vs 3.0 %	< 0.0001	Yes

Table F.6. Sidak's multiple comparisons tests between the means of *Galdieria* sp. RTK37.1 OD_{600} values at different inlet O_2 concentrations in the coculture (R3) chemostat.

Means Compared	*p*-values	Significant difference between means?
1.0 % vs 1.6 %	0.4600	No
1.0 % vs 2.1 %	< 0.0001	Yes
1.0 % vs 3.0 %	< 0.0001	Yes
1.6 % vs 2.1 %	< 0.0001	Yes
1.6 % vs 3.0 %	< 0.0001	Yes
2.1 % vs 3.0 %	0.0005	Yes

Appendix G. Time-series data for chemostat reactors

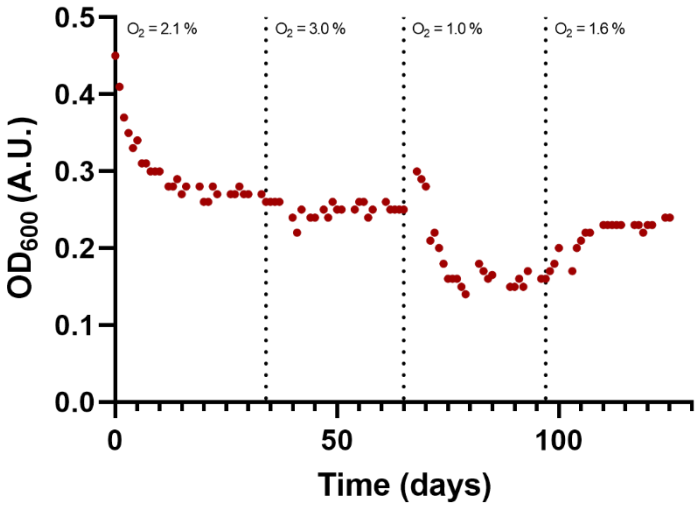

Figure G.1. OD_{600} time-series for a *Methylacidiphilum* sp. RTK17.1 axenic chemostat (D = 0.278 day^{-1}) with variable gas inlet O_2 concentrations.

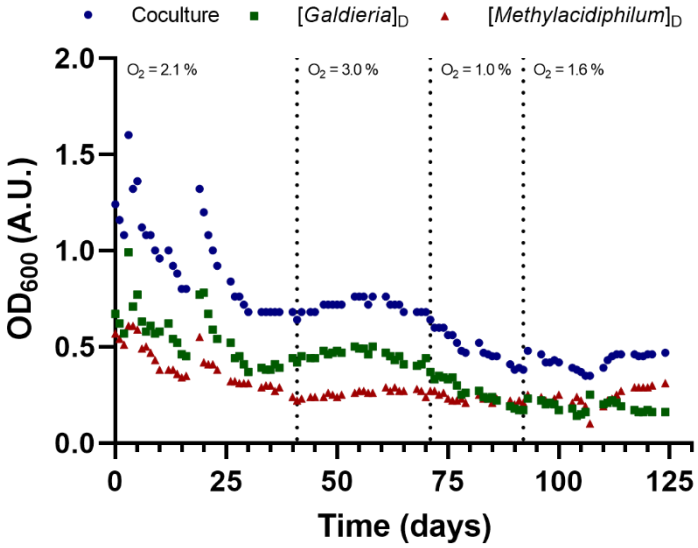

Figure G.2. OD_{600} time-series for a *Methylacidiphilum* sp. RTK17.1 and *Galdieria* sp. RTK37.1 coculture chemostat (D = 0.278 day^{-1}) with variable gas inlet O_2 concentrations.

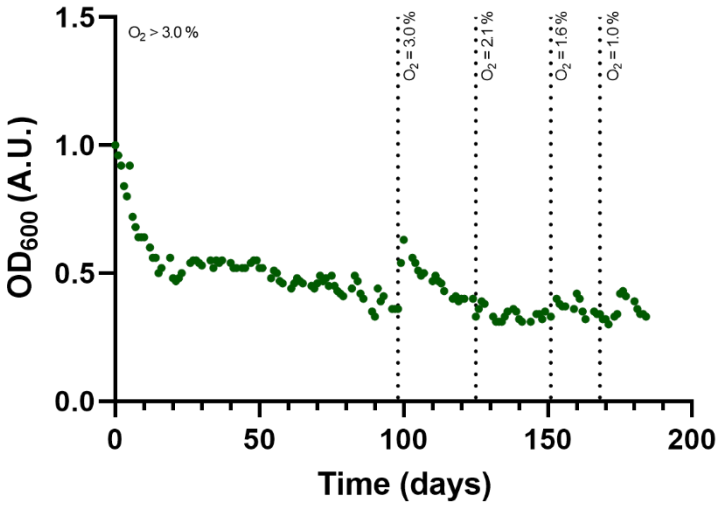

Figure G.3. OD_{600} time-series for a *Galdieria* sp. RTK37.1 axenic chemostat (D = 0.278 day^{-1}) with variable gas inlet O_2 concentrations.

Appendix H. *Methylacidiphilum* sp. RTK17.1 growth rate as a function of pressure.

Methylacidiphilum sp. RTK17.1 was cultured in a 10 L STR reactor in batch (as described in Section 2.2.4) at different pressures (Figure H.1). The results were fitted to an exponential growth model, and the growth rates calculated (Table H.1).

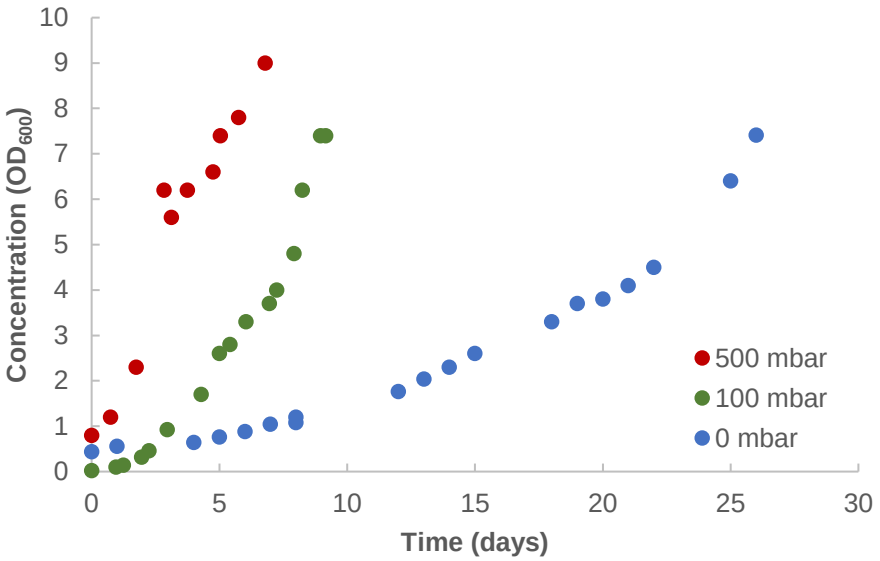

Figure H.1. *Methylacidiphilum* sp. RTK17.1 growth curves at 0, 100, and 500 mbar (gauge).

Table H.1. *Methylacidiphilum* RTK17.1 growth rate as a function of pressure in a 10 L STR reactor.

Pressure (mbar)	μ (h^{-1})	Doubling time (h)
0	0.0039	177.7
100	0.0084	82.5
500	0.0300	23.1