



Effect of different cultivation conditions of photosynthetic microorganisms on methane consumption

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e do
Doutor Pedro Miguel Macedo Geada

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Efeito de diferentes condições de crescimento de microrganismos fotossintéticos no consumo de metano

Resumo

O metano (CH_4), é o segundo gás com maior efeito de estufa na atmosfera, depois do dióxido de carbono (CO_2). Atualmente, o balanço global de CH_4 envolve diferentes fontes de emissão, equilibradas por um número inferior de fontes de absorção. Durante a COP26 foi lançado o "Global Methane Pledge" com o intuito de reduzir as emissões de CH_4 em 30% até 2030, o que torna necessário o desenvolvimento de novas e eficientes medidas de mitigação do CH_4 .

Neste trabalho, o principal objetivo consistiu no desenvolvimento de um sistema inovador e sustentável de mitigação de CH_4 com base em microrganismos fotossintéticos, reduzindo, assim, o efeito de estufa deste gás. Neste sentido, foi estudado o impacto de diferentes métodos de cultivo no consumo de CH_4 por parte de cianobactérias. Numa primeira fase, foi avaliado o efeito de diferentes concentrações de $\text{CO}_2 + \text{CH}_4$ – de 0.5 %; 1 %; 2.5 % - e apenas CH_4 - de 1 %; 2 %; 5 %; 20 % - no crescimento de *Synechocystis* sp. LEGE 07211 a 2 bar. Posteriormente, foi também realizado o estudo do efeito de diferentes variáveis – concentrações iniciais de O_2 , CO_2 e CH_4 e a proporção de cianobactéria – em parâmetros cinéticos, consumo de gases, remoção de nitratos e fosfatos e teor de azoto total em co-culturas de *Synechocystis* sp. LEGE 07211 e *Methylococcus* sp., recorrendo à metodologia de desenho de composto central (DCC).

No cultivo de *Synechocystis* sp. LEGE 07211 com diferentes concentrações de $\text{CO}_2 + \text{CH}_4$ e apenas CH_4 não se verificou consumo de CH_4 . Considerando os ensaios do desenho experimental, notou-se que o maior consumo de CH_4 por parte da co-cultura ocorre sob concentrações que tendem para 5% de CH_4 e uma proporção de cianobactérias de 25%. Além disso, também foi observado que concentrações que tendem para 5% de CO_2 , proporções mais elevadas de cianobactérias (75%) e concentrações mais baixas de CH_4 (5%) resultam em maiores consumos de CO_2 . Para uma maior remoção de nitratos, tendem a ser necessárias concentrações mais baixas de CO_2 (5%). No caso da concentração total de azoto, esta aumenta quando estão presentes concentrações mais baixas de O_2 e CO_2 , assim como menores proporções de cianobactérias. Por outro lado, concentrações mais elevadas de CH_4 (45%) também demonstraram possuir o mesmo efeito. Com base nestes resultados, é possível concluir que o co-cultivo de bactérias metanotróficas e cianobactérias promoveu a mitigação de CH_4 num sistema pressurizado. No entanto, o mesmo não se verificou para o consumo de CO_2 .

Palavras-chave: Co-culturas; Mitigação de CH_4 ; *Methylococcus* sp.; *Synechocystis* sp.

Effect of different cultivation conditions of photosynthetic microorganisms on methane consumption

Abstract

Methane (CH_4) is the second most prevalent GHG in the atmosphere, after CO_2 . Currently, the global budget of CH_4 consists of a diverse mix of sources balanced by a much lower number of sinks. During COP26 it was launched the “Global Methane Pledge” to reduce CH_4 emissions by at least 30 % by 2030, presenting a need for new and efficient CH_4 mitigation measures to be developed.

Therefore, the main goal of this thesis was to develop an innovative and sustainable CH_4 mitigation system based on photosynthetic microorganisms, thus reducing its greenhouse effect. In this work, the effect of different cultivation methods on the consumption of CH_4 by cyanobacteria were studied. *Synechocystis* sp. LEGE 07211 was tested under 2 bar with different concentrations of $\text{CO}_2 + \text{CH}_4$ – from 0.5 % to 2.5 % - and with CH_4 – from 1 % to 20 %. In addition, an experimental design (using central composite design methodology) was performed in order to evaluate the effect of different variables (initial concentration of O_2 , CO_2 , and CH_4 and ratio of cyanobacteria) on growth kinetics, gas consumption, nitrates and phosphates removal, and total nitrogen content of co-cultures of *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp.

The cultivation of *Synechocystis* sp. LEGE 07211 with different concentrations of $\text{CO}_2 + \text{CH}_4$ and CH_4 alone showed no CH_4 consumption. Considering the experimental design performed, it was observed that the CH_4 consumption by the co-culture tends to be higher under concentrations of approximately 5% of CH_4 and a ratio of 25% cyanobacteria. It was also observed that concentrations tending towards 5% CH_4 , 5% CO_2 concentrations, and higher ratios of cyanobacteria (75%) result in higher consumptions of CO_2 . For higher removal of nitrates, lower concentrations of CO_2 (5%) should be applied. On the other hand, the total nitrogen concentration tends to increase when lower initial concentrations of O_2 and CO_2 are used and lower ratios of cyanobacteria are present. Higher concentrations of CH_4 also demonstrated to have the same effect.

With these results it is possible to conclude that co-cultivation of the tested methanotrophic bacteria and cyanobacteria promoted the mitigation of CH_4 under a pressurized system. However, the same effect was not observed on the consumption of CO_2 .

Keywords: CH_4 mitigation; *Synechocystis* sp.; *Methylococcus* sp.; co-cultures

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List of abbreviations and acronyms

AD	– Anaerobic digestion
AMB	– Aerobic methanotrophic bacteria
AMS	- Ammonium mineral salts
ANME	– Anaerobic methane oxidizing archaea
ANOVA	– Analysis of variance
AOM	– Aerobic methanotrophic archaea
BG-11	– Blue-green Medium
CCD	– Central Composite Design
CoM	– Coenzyme M
COP26	- 2021 United Nations Climate Change Conference
DHA	– Docosahexaenoic
dNMS	– Diluted nitrate mineral salt medium
DOE	– Design of experiments
EDTA	– Ethylenediaminetetraacetic acid
EPA	- Eicosapentaenoic
EPn	- Ethylphosphonate
Fadh	– Formaldehyde dehydrogenase
Fdh	– Formate dehydrogenase
Fdh	– Formate dehydrogenase
Fmd	– Formyl-MF dehydrogenase
Ftr	– Formyl-MF: formyltransferase
GHG	– Greenhouse gases
GM	– Green microalgae
HEPES	- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCO ₃ ⁻	- Hydrogencarbonate
H ₂ S	– Hydrogen sulfide
Mch	– N5, N10 – methenyl-H4PT cyclohydrolase
Mcr	– Methyl-CoM reductase

MDH – Periplasmic methanol dehydrogenase

Mer – N5, N10 – methylene-H4MPT reductase

MMO – Methane monooxygenase

MPn – Methylphosphonate

MSM – Mineral salt medium

Mtd – F420, dependent N5, N10 – methylene-H4PT dehydrogenase

Mtr – N5 – methyl-H4MPT coenzyme M methyltransferase

MWO – World meteorological organisation

Methylococcus sp. – Enrichment of *Methylococcus sp*

NAD⁺ - Nicotinamide adenine dinucleotide oxidised form

NADH - Nicotinamide adenine dinucleotide reduced form

Nd – Not defined

NH₄ - Ammonium

NMS – Nitrate médium salt medium

NO₃ - Nitrate

N₂O - Nitrous oxide

NO₂ – Nitrogen dioxide

OH - Hydroxyl radicals

O₃ – Trioxygen / Ozone

P - Phosphorus

PAH – Polycyclic aromatic hydrocarbon

PBR - Photobioreactor

PHA – Polyhydroxyalkanoates

PHB - Polyhydroxybutyrate

pMMO – Particulate methane monooxygenase

PGA-2- 2- phosphoglycerate

PGA-3 – 3- phosphoglycerate

PO₄ - Phosphate

PS – Photosystem

RuBisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuMP pathway – Ribulose monophosphate pathway

sMMO – Soluble methane monooxygenase

SO₄ - Sulphate

SRB – Sulphate-reducing bacteria

TMO – Trace methane oxidation

X_{max} – Maximum biomass concentration

Z8 – Z8 medium

μ_{max} – Maximum specific growth rate

% - Per cent

€ - Euro

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1. Introduction

In recent decades, the excessive global emission of greenhouse gases (GHGs) has been one of the greatest concerns for Mankind. Massive amounts of CO₂, CH₄, NO₂, and other GHGs are emitted regardless of their well-known harmful impacts on the world's climate system and biodiversity, mainly driven by Man's greediness and desire for economic growth (Althor et al., 2016). In 2015, the Paris Agreement was a landmark of the Global commitment to reduce GHGs emissions. However, recent studies have shown new atmospheric concentration highs for CO₂ (415.8 ± 0.2 ppm), CH₄ (1908 ± 2 ppb), and N₂O (334.5 ± 0.1 ppb) in 2021 (World Meteorological Organisation (MWO), 2022).

Methane (CH₄) is the second most prevalent GHG in the atmosphere resulting from anthropogenic activities, presenting, in 2021, 260% of the pre-industrial levels. Its global warming potential represents a small fraction of the importance of its reduction in the atmosphere (Smith et al., 2010). CH₄ has a perturbation lifetime of 12.4 years, while the emission of CO₂ requires 37 years to be partially eliminated from the atmosphere. Additionally, CH₄ emissions are much smaller than those of CO₂. However, CH₄ is more efficient on trapping radiation, presenting 28-36 times the global warming potential of CO₂ per unit of mass in a 100-year time scale. Also, CH₄ decay leads to tropospheric O₃ and stratospheric H₂O formation, aggravating its radiative forcing (energy imbalance available to the climate system caused by a change in the components of the Earth's radiative budget) and causing global warming and catastrophic climate changes (Miller et al., 2013; IPCC, 2014; Olczak & Piebalgs, 2019; World Meteorological Organisation (MWO), 2022). Besides the potential to increase the formation of surface ozone (a major component of smog) in populated regions, leading to several health problems for humans, CH₄ can also affect the ability of the atmosphere to oxidize other pollutants. Therefore, in summary, reducing CH₄ emissions has become imperative because of its effect on warming rates in the near term (UNEP, 2021).

1.1. Sources and sinks of CH₄

Nowadays, the global budget of CH₄ in the atmosphere consists of a diverse mix of sources balanced by a much lower number of sinks. In fact, the levels of CH₄ have increased significantly due to the combination of natural and anthropogenic sources - with particular importance of the latter - and are likely to continue increasing globally (Miller et al., 2013; Van Dingenen et al., 2018).

1.1.1. Anthropogenic sources

Anthropogenic sources represent 60% of the total CH₄ released to the atmosphere. These emissions are caused by human activities of different sectors, as mentioned in Table 1. Agriculture and cattle raising, waste, and energy are some of the causes of the high CH₄ emissions released in the last century.

Table 1. Main sectors responsible for anthropogenic CH₄ emissions (Karakurt et al., 2012; Olczak & Piebalgs, 2019)

Agriculture and cattle raising (237 MtCO ₂ eq)	Waste (124 MtCO ₂ eq)	Energy (85 MtCO ₂ eq)
Rice cultivation	Landfills and solid waste	Biomass combustion
Manure management	Waste combustion	Coal Mining
Enteric Fermentation	Wastewater	Gas, oil industry

a) Agriculture and cattle raising

Agriculture and cattle raising sector is considered the major source of anthropogenic CH₄, representing 237 MtCO₂eq in 2018 (Olczak & Piebalgs, 2019). In this sector, CH₄ is generated mainly by ruminants through enteric fermentation. This process is known as the feed fermentation that occurs in animals' digestive system resulting in the release of CH₄ to the atmosphere as a by-product (Karakurt et al., 2012). Rice fields frequently produce anoxic and carbon-rich conditions that allow microbial methanogenesis (process that produces CH₄) to occur (Islam et al., 2018). Methanogenesis may also occur when manure is stored or treated (Karakurt et al., 2012).

b) Waste

Waste represents the second biggest contributor to CH₄ emissions produced by humans. Solid waste and wastewater are the most relevant emission sources, followed by waste combustion. In 2012, wastewater and solid waste represented 69 Tg CH₄ year⁻¹ (19% of the global total antropogenic CH₄) (Van Dingenen et al., 2018). As happens in the case of rice fields, landfills also provide anoxic conditions suitable for methanogenesis, making them a great source of CH₄. Most of the solid waste present in these sites is municipal waste, although agricultural waste and sewage sludge are also commonly incorporated, exacerbating the problem (Smith et al., 2010).

c) Energy

This sector is responsible for the emission of 85 MtCO₂eq of anthropogenic CH₄ (Olczak & Piebalgs, 2019). Most of these emissions result from gas and oil industry, followed by coal mining and biomass combustion. Studies indicate that over 80% of the emissions in the energy sector are result of fugitive emissions from coal mining and production, processing, transmission, storage, and distribution processes of natural gas (Karakurt et al., 2012; Olczak & Piebalgs, 2019). Recently, natural gas has gathered increasing attention as a “clean” burning fuel for electricity generation and vehicles power, being its use associated with reduced air pollution in cities. However, it is important to note that 95% of natural gas is composed of CH₄ and is generated from non-renewable energy sources (Johannisson & Hiete, 2020).

Biomass combustion represents a very small percentage of emissions when compared with the rest of the activities in this sector. Nonetheless, they are still considerable. The most commonly incomplete biomass combustion happens through fuel wood, charcoal, agricultural residues and waste, and municipal waste combustion, where CH₄ is released to the atmosphere (Grass & Jenkins, 1994).

1.1.2. Natural sources

Natural sources represent just 40 % of the total CH₄ released to the atmosphere, meaning they are currently outweighed by anthropogenic sources (WMO, 2019). These include natural wetlands, wild animals, wildfires, termites, oceans, hydrates, permafrost, lakes, rivers, and vegetation. In regard to wildfires, it is important to note that these differ from fires caused by human activities, even though it is often difficult to differentiate both of them. With respect to hydrates, the opinions about CH₄ emissions are still very unclear. Some reports showed that they are responsible for emissions of 4-5 Tg of CH₄ year⁻¹, whereas there are others estimating quite greater and diverse emissions (40 to 60 Tg CH₄ year⁻¹), highlighting the big losses that may happen through seeps, mud volcanoes and geothermal/volcanic areas (Smith et al., 2010).

1.1.3. Sinks

Regarding sinks to regulate the atmospheric concentrations of CH₄, the oxidation of CH₄ by hydroxyl radicals (OH) is the primary sink, destroying up to 90% of CH₄ in the troposphere. The remaining sinks are associated with CH₄ oxidizing microorganisms, which are discussed in more detail in section 1.3.1, and chemical oxidation by chlorine in the air and on seas' surface (Kirschke et al., 2013).

1.1.4. Methane Paradox

Lately, it has been observed an oversaturation of CH_4 in mid-water of the oceans and lakes, often referred to as the methane paradox. This has been debated for several years since CH_4 production was believed to occur only under anoxic conditions (Ordóñez et al., 2023; Tang et al., 2014). However, numerous studies have shown an increase of this phenomenon and many theories have been proposed. Hofmann et al. (2010) suggested that nearby anoxic waters and sediments originated from littoral zones could mix with near surface oxic waters from pelagic zones, enhancing the flux of methane in those areas. Moreover, the existence of micro-anoxic zones (e.g., fecal pellets, gastrointestinal tracts) has also been proposed to explain this CH_4 increase (Tang et al., 2014). Another explanation is that methanogens are oxygen tolerant and could simply produce CH_4 under these oxic conditions. Repeta et al. (2016) also suggested that bacterial degradation of dissolved organic matter phosphonates under aerobic conditions releases CH_4 and could explain this phenomenon. Furthermore, it has also been shown that photoautotrophs are linked to greater CH_4 concentrations in these environments as it was found that the oversaturation of dissolved oxygen and high peaks of chlorophyll a (Chla) in Lake Stechlin matched the oversaturation of CH_4 (Grossart et al., 2011; Tang et al., 2014). Nevertheless, there is still doubt of how the interaction is achieved. The production of CH_4 has also been associated with the demethylation of methylphosphonate (MPn) and ethylphosphonate (EPn) in microalgae and cyanobacteria under phosphorous (P) starvation. In this case, when low inorganic P is available, these marine organisms use phosphonates (organic P) as source of P. This metabolism shift happens due to the activation of both the phosphonate transporter complex and the multisubunit C-P lyase complex, which cleaves the C-P bond, resulting in P that is then assimilated. This reaction also liberates CH_4 or C_2H_6 , depending on which phosphonate is being used (MPn and EPn, respectively) (Beverdorf et al., 2010). With all this in mind, cyanobacteria and microalgae could explain the increasing concentrations of CH_4 in marine environments. Additionally, the production of this GHG has been associated with a vast spectrum of cyanobacteria species under light/dark and anoxic/oxic conditions (Bižić et al. 2020). The most well-studied species regarding this process are *Synechococcus sp.*, *Microcystis sp.*, *Nodularia sp.*, *Trichodesmium sp.*, and *Phormidium sp.* (Bižić et al., 2020; Gomez-Garcia et al., 2011; Teikari et al., 2018). For instance, Bižić et al. (2020) reported the production of CH_4 in many different species of cyanobacteria/microalgae and concluded that their production rates were three to four times lower when compared to those of methanogenic archaea under ideal conditions (anoxic conditions). However, when compared to eukaryotes, these rates are one to three times higher. Some studies demonstrated a close link between

photosynthesis and CH₄ production, as CH₄ increase was aligned with light incubation periods and higher light intensities enhanced CH₄ production (Bižić et al., 2020; Günthel et al., 2020). Nevertheless, these production processes are still very debated and theorized.

1.2. Methanogenetic organisms

Methanogenic archaea (methanogens) are part of the phylum Euryarchaeota and can be found in a wide range of environments, including marine and freshwater sediments, flooded soils, human and animal gastrointestinal tracts, termites, anaerobic digestors, landfills, geothermal systems, and the heartwood of trees (Liu & Whitman, 2008). Methanogenesis is the process of CH₄ production carried out by these organisms that allows them to obtain energy for growth under anaerobic conditions. It is important to note that these microorganisms are a large and diverse group of strict anaerobes and have an important role in the global carbon cycle (Buan, 2018)

Methanogens use substrates like CO₂, methyl-group containing compounds, and acetate to produce CH₄ and, depending on the substrate used, they can take different pathways. Organic substances like carbohydrates, long-chain fatty acids, and alcohols are not substrates for methanogenesis; however, most methanogenic environments have anaerobic bacteria or eukaryotes that transform these compounds into usable substrates for methanogenic archaea (Buan, 2018).

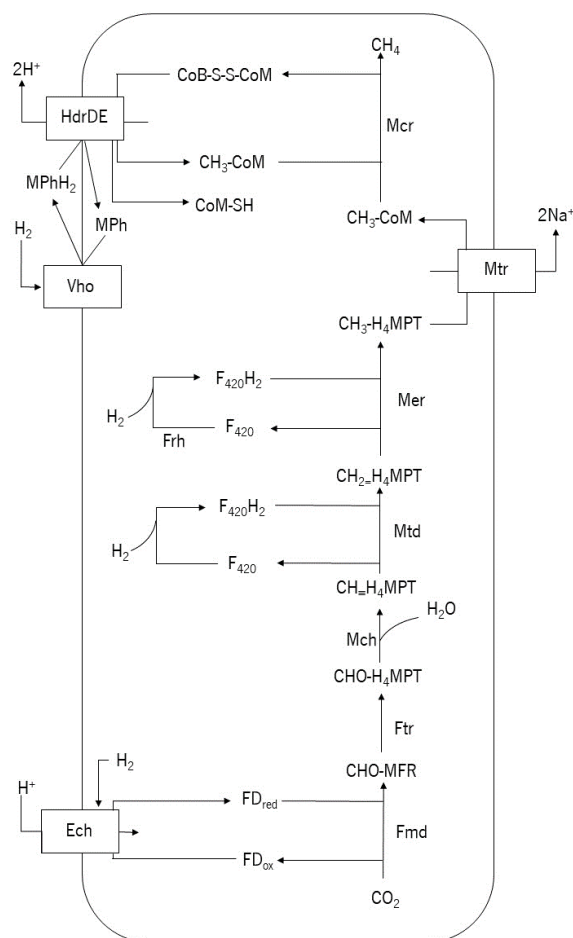


Figure 1. Overview of the hydrogenotrophic methanogenesis metabolism. Key enzymes are: Fmd (formyl-MF dehydrogenase), Ftr (formyl-MF:H4MPT formyltransferase), Mch (N5, N10-methenyl-H4MPT cyclohydrolase), Mtd (F420- dependent N5, N10-methylene - H4MPT dehydrogenase), Mer (N5, N10-methylene-H4MPT reductase), Mtr (N5-methyl-H4MPT:coenzyme M methyltransferase), Mcr (methyl-CoM reductase). Based on Timmers et al. (2017)

As stated by Liu & Whitman (2008), most of the methanogenic archaea (*Methanobacteriales* and *Methanococcales*) are hydrogenotrophs – known to reduce CO₂ using H₂ as the primary electron donor – , while others can use formate as electron donor. In this particular case, four molecules of formate are oxidized to CO₂ by formate dehydrogenase (Fdh) before one molecule of CO₂ is reduced to CH₄ (Liu & Whitman, 2008). The overview of the hydrogenotrophic methanogenesis pathway is represented in Figure 1. and the general reaction performed by most methanogens is represented in Eq 1 (Liu & Whitman, 2008).



It is important to note that some methanogenic archaea can also perform a process called trace methane oxidation (TMO), under anoxic conditions, otherwise known as reverse methanogenesis.

However, this process of complete reversal of methanogenesis is believed to occur only under stress conditions, causing the release of CO₂ and formation of reduced by-products. In this case, methanogens can oxidize methane during its production but at very small rates (Timmers et al., 2017).

1.3. Mitigation strategies

Despite the concerning issues associated with the increasing release of CH₄, CO₂ remains the focus of GHG studies, representing the basis of climate policies, which leaves, sometimes, CH₄ behind. However, during the COP26, in November 2021, it was launched the “Global Methane Pledge”, which is an action plan to reduce CH₄ emissions by at least 30% below 2020 levels by 2030 (IEA., 2022). This action plan has been signed by 150 countries since it was introduced in order to provide a net cooling effect within a relatively short period. This represents the starting point of a new era of efforts addressing CH₄ mitigation, though the establishment of new and efficient CH₄ mitigation measures is still crucial.

As CH₄ mitigation strategies mainly focus on preventing anthropogenic emissions, natural emissions strategies seem to follow behind, even though a reduction of these emissions is still possible to achieve (Stolaro et al., 2012). According to Johannisson & Hiete (2020), the mitigation of natural sources can be categorised in three groups: pre-generation mitigation, *in situ* mitigation, and post-emission mitigation. Pre-generation mitigation refers to fully or partly prevent CH₄ generation. This is achieved by adding oxygen (O₂) to the anaerobic environment needed for archaeal CH₄ generation. Currently, this is the most used mitigation strategy because of its cost-effectiveness, simplicity, and low risk of creating explosive mixtures. *In situ* mitigation implies mitigating CH₄ near the temporal and geographic point of generation. This can be achieved through extraction of CH₄ from coal mines directly from the coal seam for energy generation, mitigation of fugitive emissions, leakage detention and more. However, it is important to note that these techniques are only applied if pre-generation mitigation is impossible or very expensive. Lastly, post-emission mitigation techniques are the least studied since capturing CH₄ is far more challenging and expensive. Recent studies reveal that there are only a few options available for mitigating natural emissions and these cannot be applied in large scale since a lot of disadvantages are implied, like extensive areas of emission and significant ecological effects. However, some mitigation options could be drainage or restoration of natural wetlands, increasing herbivore density in permafrost thawing, bubble management and lake sealing in freshwater systems, although further studies are needed to assess the feasibility of all these options (Johannisson & Hiete, 2020).

Many strategies for anthropogenic CH₄ emissions mitigation have been studied; however, they present many disadvantages, such as high costs or the release of non-desirable by-products.

Consequently, even though the potential application of different strategies and technologies to reduce CH₄ release has been proven, few mitigation approaches are in fact in practice (Beauchemin et al., 2020; Hopkins et al., 2016).

According to Beauchemin et al. (2020), the effort on the livestock management has recently been focused on improving the rumen fermentation efficiency while increasing productivity of dairy animals. For these purposes, genetic selection, vaccination, and diet changes using direct inhibitors, feed additives, propionate enhancers, CH₄ oxidizers, probiotics, defaunation, and hormones can be used. Recently, a study has shown that using red macroalgae *Asparagopsis* spp. as feed additive could decrease ruminant enteric CH₄ production up to 99 % in vitro (Id et al., 2021). Nevertheless, there are some concerns regarding the toxicity of this macroalga since this organism is capable of synthesizing inorganic halogen compounds, such as bromoform, which should be carefully monitored (Eason & Fennessy, 2023). However, it is still unknown if CH₄ emissions could have an impactful reduction at larger scale. Therefore, an environmental impact assessment is still needed to evaluate all the aforementioned strategies. Furthermore, these approaches require additional costs that might make them unfeasible to implement (Beauchemin et al., 2020; Karakurt et al., 2012; Van Dingenen et al., 2018). Reports show that combinations of anti-methanogenic compounds with complementary modes of action are a promising approach to achieve effective CH₄ mitigation without adverse effects on feed intake and rumen fermentation (Patra et al., 2017). For manure management, switching to dry manure is also a possibility. However, the aerobic conditions used during manure storage can promote the formation of nitrous oxide (N₂O), jeopardizing the purpose of reducing GHGs (Bosello et al., 2011; Karakurt et al., 2012). In rice cultivation, the main strategies used are: *i*) upland rice cultivation; *ii*) cultivars with lower exudation rates; and *iii*) water management through draining wetlands during rice seasons, avoiding waterlogging in off-seasons and shallow flooding; *iv*) fertilizer management and use of nitrification inhibitors; *v*) application of biochar. Nonetheless, as happens in the case of manure management, some of these practices might lead to the increase of N₂O (Bosello et al., 2011; Gummert et al., 2019).

Preventing CH₄ production is the most used strategy for wastewater management. As stated by Karakurt et al. (2012), this can be achieved using aerobic primary and secondary treatment and land treatment. Anaerobic conditions might also be applied where the generated CH₄ can be used as fuel, to generate electricity, or captured and flared, thus oxidizing CH₄ into CO₂ and causing another GHG mitigation problem.

CH₄ produced in landfills can be captured through vertical wells or horizontal trenches and can be used directly as a gas or for energy as local heat and/or electricity generation. Besides this, the remaining CH₄ can also be oxidized into CO₂ through flaring or methanotrophic microorganisms present in landfill cover soils (Bosello et al., 2011).

Based on Johannisson & Hiete (2020), the most effective way for mitigation in the oil and gas sector is through progressively eliminating the use of fossil resources and switch to renewable energy sources. Biogas is an example of a widely used renewable source, normally composed of 40-75 % CH₄, 15-50 % CO₂, and other components such as O₂ (0 - 1 %), N₂ (0 - 2 %), water vapour (1% - 10 %), H₂S (0-10.000 ppm), ammonia (0 - 100 ppm), siloxanes (0 - 0.02 %), and halogenated hydrocarbons (VOC < 0.6%). It is produced through anaerobic digestion (AD) of different biomass (poultry droppings, agricultural crop wastes, and cattle manure), which benefits include production of a renewable energy resource while leading to useful organic fertilizers that can substitute chemical fertilizers (Kabeyi & Olanrewaju, 2022).

It is important to note that CH₄ mitigation doesn't always imply eliminating completely the emissions of CH₄, as cleaning and upgrading paths of CH₄ can be used for further industrial usage. In fact, cleaning and upgrading raw biogas into biomethane has been recently studied since high-purity CH₄ biogas has very similar heating properties to natural gas, while still using carbon-neutral energy sources (biogas) and allows for H₂S – a corrosive compound – removal. Different methods can be used to achieve biomethane (95 - 99% CH₄), from chemical to physical; however, high amounts of energy and harsh chemicals are usually involved. Chemical scrubbing is a widely used methodology for biomethane obtention that requires a heat demand of 100 °C - 180 °C as well as a running cost of 134,500 € and an investment of 353,000 € (Khan et al., 2021). According to a recent report, the average global cost of upgrading biogas to biomethane costs around 60.8 €/MWh. Though, most of this cost is attributed to the production of the biogas itself, since the upgrading process cost is around 6.4 €/MWh and 12.8 €/MWh for facilities (IEA, 2020). Biological technologies play a critical role in biomethane production since it doesn't entail such high costs, having the advantage of extra value biomass and high-purity CH₄-biogas (Khan et al., 2021). Methanogens can be used in order reduce the CO₂ present in the biogas and release CH₄, leading to a significant increase CH₄ purity (Adnan et al., 2019). Moreover, microalgae and cyanobacteria have also demonstrated to be efficient in upgrading biogas to biomethane (Khan et al., 2021; Nagarajan et al., 2019). Another study has shown that microalgae could remove CO₂ with efficiencies higher than 80%, providing a biomethane with concentrations of CH₄ of approximately 90% (Muñoz et al., 2015). In summary, biogas upgrading using biological methods can serve as a carbon sequestration strategy, supported by a circular economy approach where high-value biomass is produced.

1.3.1. Methanotrophic bacteria

Methanotrophic bacteria are a group of gram-negative bacteria and archaea. These organisms are part of a larger group called methylotrophs that use/reduce carbon from various methyl compounds (methanol, methylated amines, halomethanes, and CH_4). They can also be found in a wide range of environments such as soils, peatlands, rice paddies, sediments, freshwater, marine systems, acidic hot springs, mud pots, alkaline soda lakes, cold environments, and tissues of higher organisms (Smith, 2009).

In fact, methanotrophs are a group of aerobic microorganisms that possess the unique capability to use CH_4 as their source of carbon and energy (Hanson & Hanson, 1996; Smith, 2009). Although the general pathway is a very simple oxidation step of CH_4 to CO_2 , a lot of important intermediates and enzymatic systems are present, giving a lot of complexity to the process (Figure 2).

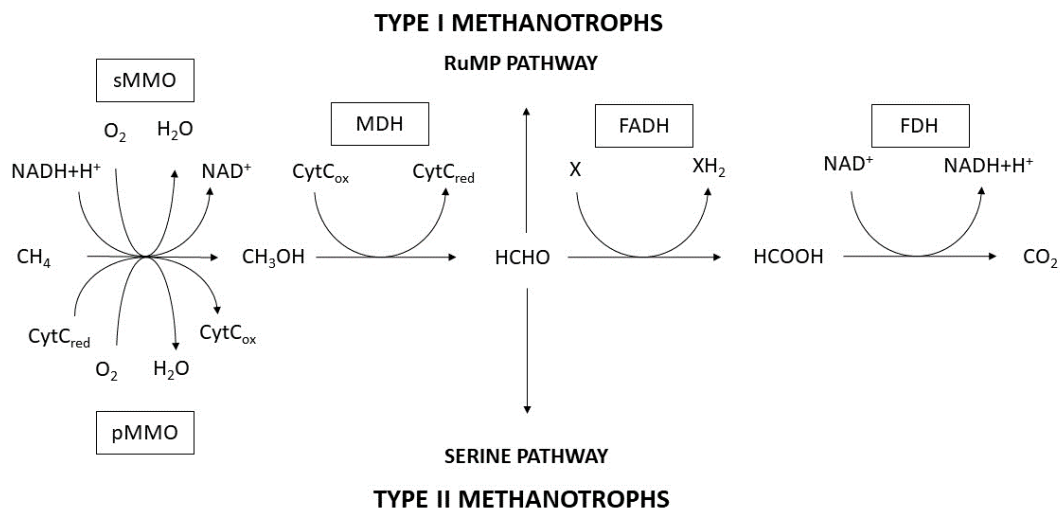


Figure 2. Overview of the methanotrophic metabolism and assimilation of formaldehyde. Key enzymes are: pMMO: particulate methane monooxygenase; sMMO: soluble methane monooxygenase; Mdh: methanol dehydrogenase; FadH: formaldehyde dehydrogenase; Fdh: formate dehydrogenase. Based on Hanson & Hanson, (1996)

In fact, a specific characteristic of methanotrophs is the usage of the enzyme methane monooxygenase (MMO) to catalyse the oxidation of CH_4 to methanol in the presence of O_2 (Semrau et al., 2010). This highlights the extreme importance of O_2 in this process. For this conversion, two forms of MMO have been found: membrane-associated or particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO), which are encoded by the genes *pmoA* and *mmoX*, respectively. It has been shown that methanotrophs presenting pMMO have higher growth yields when exposed to CH_4 and greater affinity to CH_4 than those that have sMMO (Hanson & Hanson, 1996). Besides this, copper (Cu) has been reported as a key regulator of the expression and activity of both forms of

MMO. Though, the impact of Cu seems differ since the gene responsible for sMMO is only expressed when Cu concentrations are low (<1 μM), while *pmoA* expression is improved under high-Cu environments (Semrau et al., 2010). According to Khmelenina et al. (2019), methane oxidation via MMO is followed by methanol oxidation via MDH (periplasmic methanol dehydrogenase), forming formaldehyde. Partially of this compound is oxidized to CO_2 by a formaldehyde dehydrogenase and a NAD-dependent formate dehydrogenase, releasing NADH that can be further used for methane oxidation. The rest of this compound is then used as carbon source via RuMP pathway or serine pathway. The general reaction of methanotrophic bacteria, as well as its Gibbs free energy, is represented in Eq 2 (Yao et al., 2023).



It is important to note that these bacteria can be classified into three groups (type I, II, and X) based on the pathways used for assimilation of formaldehyde, the main source of carbon, and other physiological and morphological features. Type I and type X are part of Gammaproteobacteria family, which use ribulose monophosphate pathway for formaldehyde assimilation, whilst type II (Alphaproteobacteria family) use serine pathway for the same purpose. However, type X has been found to be able to use this last pathway as well under certain conditions (Hanson & Hanson, 1996). Additionally, Type I methanotrophs use pMMO, while type II methanotrophs produce sMMO in addition to pMMO to carry out the CH_4 oxidation process (Hanson & Hanson, 1996). Moreover, environments with high levels of CH_4 , low levels of dissolved oxygen and limiting concentrations of nitrogen and/or copper benefit the growth of type II bacteria, whereas those with limited CH_4 and high concentrations of nitrogen and copper favour type I bacteria. Type X has been found to survive under extreme temperatures and pH levels, which are less favourable for the other two types of methanotrophs (Zappi et al., 2020).

Methylococcus geothermalis is a type I methanotroph and strict aerobe that has been recently discovered (Awala et al., 2020). This species of methanotroph uses only CH_4 and methanol as substrates to obtain energy. According to Awala et al. (2020), this microorganism is capable of growing between 15 and 48 $^{\circ}\text{C}$ (optimum, 30–45 $^{\circ}\text{C}$) and pH values between 4.8 and 8.2 (optimum, pH 6.2–7.0). Moreover, nitrogen sources include ammonium (sulphate and chloride), nitrate, urea, yeast extract, L-tryptophan, L-glutamine, glycine, alanine, and dinitrogen gas. It is important to note that due to the novelty of this species' discovery, little information is available yet.

It is important to reinforce the existence of other methanotrophic organisms, such as methanotrophic archaea (AOM), sulphate-reducing bacteria, and nitrite-reducing bacteria, which are capable of performing anaerobic oxidation of methane (Guerrero-Cruz et al., 2021). Moreover, some

microorganisms – ANME (anaerobic methane oxidizing archaea) – were found near CH₄ seeps and on oceans CH₄ hydrate deposits that are also believed to perform reverse methanogenesis. However, this is still a hypothesis to be proven (Scheller et al., 2010).

1.3.1.1. Cultivation strategies

Regarding the cultivation process of methanotrophic bacteria, mixing strategies and reactor designs are the major bottlenecks and despite the increasing number of studies describing the metabolism and products, little attention is given to the design and operating features of bioreactors used for cultivation (Tikhomirova & But, 2021). The reactors require detailed analysis of stirring, gas supply, construction, and maintenance costs as they tend to be very high. Additionally, extraction methods of metabolites are also a potential obstacle since they are expensive, require high temperatures and pressure and some of them are toxic. Regarding the cultivation of methanotrophic bacteria industrially, there is a lack of research and understanding as most of the studies are carried out at lab-scale. Thus, a shortage of information about the viability of these processes marks a major bottleneck when it comes to scale-up the production of these organisms. However, one species in particular has been tested at this scale (*Methylococcus capsulatus*), mainly due to its fast growth rate, genetic manipulation ability, and viability (Zappi et al., 2020).

Gas supply is a very important parameter when culturing methanotrophic bacteria. CH₄ has low solubility in water (22 mg/L (25°C), contrarily to CO₂ (42 mg/L (25°C)), which causes limited exposure of this GHG to the culture. Since this is crucial to an adequate growth, careful analysis should be done when selecting and developing bioreactor designs (Zappi et al., 2020). Regardless of the atmosphere utilized, methanotrophic bacteria can grow in various media (e.g., NMS, AMS) under batch, continuous and fed-batch systems, greatly depending on its future use (Awala et al., 2020; Jiang et al., 2010; Zappi et al., 2020). Usually, valuable products obtained by these organisms (e.g., ectoine) are produced under fed-batch approaches; however, these processes are very expensive due to the high-quality substrates required and complex downstream processing. Therefore, new, and innovative alternatives are still needed to improve these processes (Cantera et al., 2018).

Methanotrophic bacteria can grow both in pure cultures and mixed cultures; however, different growth rates are obtained. In pure cultures, low growth rates are usually achieved, whilst mixed cultures can grow more rapidly. The latter can be achieved with the intention, for example, to enrich a culture, using growing on nitrate mineral salts (NMS) medium with CH₄ and high concentrations of iron. Depending on the type of organism (I, II or X), low or high copper concentrations can be used. This provides high

cell density cultures and the appropriate MMO enzyme activity. Additionally, the mixed cultures can be used to create a synergy between the organisms in order to eliminate or produce a specific substrate or by-product, respectively. The consortia can be formed by different methanotrophs or other microorganisms like microalgae and cyanobacteria and methanotrophs, which perform well in co-metabolism (Hill et al., 2017; Jiang et al., 2010)

1.3.1.1.1. Pressurized systems

As mentioned in section 1.3.1.1. gas supply and mass transfer rates of gases are very critical factors when culturing methanotrophic bacteria, especially due to the low solubility of CH₄ in aqueous solution. The solubility of this gas is highly dependent on various factors like homogenization, bioreactor type and its mechanical design, composition of the medium, and the possible effect of the presence of microorganisms. Therefore, the use of optimized conditions, such as temperature, pH, pressure, mixing, and concentration of biomass and nutrients, allows for higher mass transfer rates (Cantera et al., 2018; Guerrero-Cruz et al., 2021; Tikhomirova & But, 2021). In fact, CH₄ solubility is highly dependent on pressure and temperature since higher pressures and lower temperatures allow for higher dissolution of this gas, which allows the cells to have more substrate to use. However, lower temperatures are difficult to achieve when working with living organisms; therefore, pressure is one of the mostly used strategies to increase CH₄ mass transfer rate. Many studies have proven that pressurized systems enable, in fact, higher growth rates of methanotrophic bacteria. Wendlandt et al. (1993) showed how *Methylocystis* sp. GB 25 behaved in continuous and discontinuous processes under the pressure of 5 bar. In this study, a fermenter of 12 L allowed for a maximum productivity of 6 g biomass. L⁻¹h⁻¹ mainly due to the increase in the concentration of dissolved O₂, CO₂, and CH₄. Moreover, Nauhaus et al. (2002) also demonstrated that pressurized systems (11 bar) could be used to enhance a consortium of sulphate-reducing bacteria (SRB) and AOM and enable high activity rates by guaranteeing the availability and saturation of CH₄, in comparison to atmospheric conditions – pressure of 1 bar.

It is important to note that other alternatives, such as the addition of paraffin and nanoparticles, have shown to increase mass transfer rate of CH₄. However, high costs are associated with these methods. Moreover, many types of reactors, as the case of continuous stirred tank reactors, bubble columns, airlift reactors, membrane-fed reactors, sequencing batch reactors, trickle beds reactors, and two-phase partition bioreactors, have also been widely explored to improve gas transfer efficiency, as well

as their combined use with a pressurized headspace (Figure 5)(Guerrero-Cruz et al., 2021; Rocha-Rios et al., 2010; Strong et al., 2015).

The design of pressurized reactors with CH₄ can be a difficult task since many variables must be considered in order to increase mass transfer. There has been a wide range of reactor types used at lab-scale. Currently, most studies devoted to methanotrophic bacteria/archaea focus on simple batch reactors (such as bottles, shake flasks, tanks, or fermenters) whether using a static head or flow through of gas under static, shaken or stirred conditions (Figure 5) (Guerrero-Cruz et al., 2021).

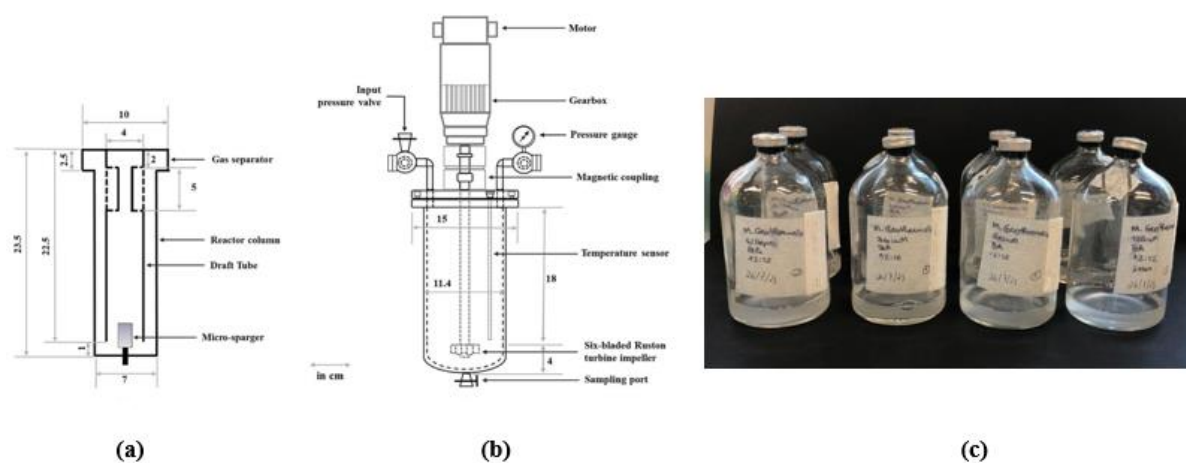


Figure 3. Examples of reactors used in methanotrophic bacteria growth: (a) internal loop airlift reactor; (b) high-pressure stirred tank reactor; and (c) pressurized bottle. Based on Sahoo et al., (2023).

In terms of large-scale, K. D. Wendlandt et al. (2001) studied the production of polyhydroxybutyrate (PHB) using a batch cultivation with a methanotroph in 7-L and 70-L reactors with a pressure of 6 bar and 20% of CH₄. Moreover, Levett et al. (2016) presented a study of a large-scale continuous bioreactor designed for methanotrophic bacteria to produce PHA. In this study, a two-stage cultivation and accumulation technique were tested using a mixed methanotrophic culture and the reactor was managed under high pressure (5 bar) with 2 % (v/v) of CH₄. A study was also conducted using a pressurized (1.4 bar) stirred tank reactor inoculated with *Actinobacillus succinogenes*, which upgraded biogas from 60 to 95.4 % of CH₄ using glucose as carbon and energy source (Muñoz et al., 2015). However, for co-cultures and microalgae/cyanobacteria cultivated in biogas, there are not many studies involving pressure, as most studies use an inert gas like nitrogen to depressurize the batch reactor before pressurizing with biogas/the gas under study at 1 bar. Generally, the goal of these studies using only microalgae/cyanobacteria is to produce biomethane. Once the carbon source for microalgae (CO₂) has a very high mass transfer rate in aqueous solutions, there is no particular reason to use high pressures to

overcome the issue of dissolution. Moreover, it is important to note that sometimes there is a lack of clarity in the scientific studies regarding microalgae and co-culture processes when it comes to show if the experiments are done using pressurized headspace or just by adding small quantities of the desired gas. Therefore, this should be uniformized for better understanding of the work under evaluation.

1.3.1.2. Applications and commercial value

Methanotrophs play a key role in the carbon cycle by using CH₄ as a carbon and energy source. However, their usage is mainly limited to environmental applications. With this, their restricted market applications are the main disadvantage, causing a major bottleneck on their widespread use (Semrau et al., 2010). Currently, methanotrophic bacteria have been used as a natural catalyser to produce high-value compounds such as methanol, formaldehyde, short chain fatty acids, volatile organic compounds (e.g., lactate, acetate), ectoine, and biopolymers. Biodiesel production through membrane lipids that these microorganisms retain is a very promising biotechnological application as well. Additionally, many methanotrophic bacteria can accumulate up to 90% of polyhydroxy alkanates (PHAs) under specific conditions, representing a lucrative approach to produce bioplastics (Guerrero-Cruz et al., 2021). Another potential application of these organisms is organic pollutant degradation and methane mitigation in the many of the sectors mentioned above (Semrau & Dispirito, 2019). Moreover, methanotrophic bacteria have been used as feed and supplements for chicken, salmon, minks, and dogs (Semrau et al., 2010). Methanotrophic biomass can also generate single-cell proteins (SCP), which can be considered nutritive proteins comparable to common protein sources (e.g., fish, soy) at lower cost (Rasouli et al., 2018).

1.3.2. Cyanobacteria and Microalgae

Microalgae are unicellular or multicellular eukaryotic microorganisms, while cyanobacteria are prokaryotic organisms. Both these organisms can thrive in many different environments (marine, freshwater, and terrestrial) and it is roughly estimated that more than 44,000 species of microalgae and cyanobacteria are currently scientifically described (Novoveská, et al., 2023).

Microalgae and cyanobacteria are photoautotrophs, which means they can use light, water, and CO₂ to produce biomass through the photosynthesis process. However, many species can also grow heterotrophically using several sources of organic carbon or mixotrophically. In autotrophic growth, cells capture light (natural or artificial) and use CO₂ as carbon source. Currently, this is the most commonly used regime for microalgae growth since it is an easy approach and results in both CO₂ mitigation and synthesis of high-value metabolites (e.g., pigments, lipids, proteins) (Mata et al., 2010).

1.3.2.1. Cultivation systems

Currently, microalgae and cyanobacteria are produced in a broad range of cultivation systems at industrial scale (outdoors or indoors), such as open ponds and photobioreactors (PBRs) (Novoveská, et al (2023) (Figure 3.). Open ponds are usually 0.3 m deep, providing enough sunlight for photosynthesis. The culture is kept in circulation in a closed channel and the mixing is guaranteed by a mechanical arm stirring in a circular motion in circular ponds. Although these systems are an inexpensive and simple approach that provides advantages to the industrial implementation, it requires large areas and are often exposed to harsh weather conditions and microbial contaminations. Additionally, these systems use CO₂ less efficiently as they entail significant losses to the atmosphere, which reduces biomass productivity increasing the variability of the final product (Enzing et al., 2014). On the contrary, PBRs are more robust and significantly more expensive. Tubular systems of different sizes (placed either vertically or horizontally), polyethylene sleeves or bags, and flat panels are the most common. These systems are usually applied in food and pharmaceutical industries to produce high-value products since they can prevent or minimize contaminations, offer better control over cultivation conditions, prevent evaporation, and reduce water use. At the same time, they allow production of higher cell concentrations, which increases volumetric productivity (Enzing et al., 2014).

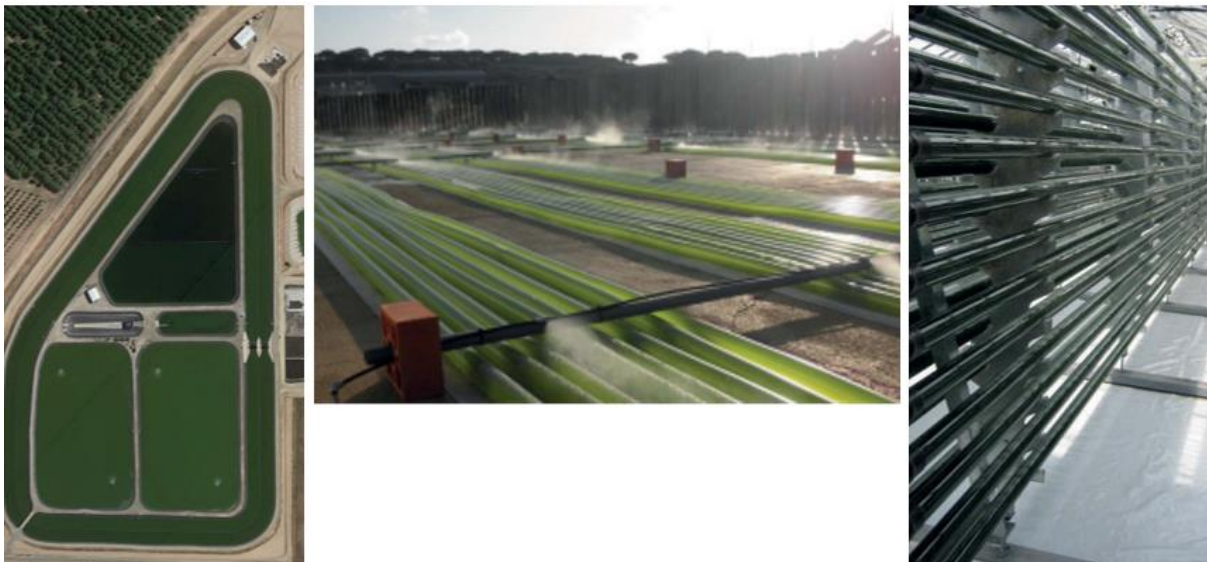


Figure 4. Examples of open ponds and tubular photobioreactors (horizontal and vertical). Based on Benemann et al., 2018; Enzing et al., 2014.

At lab-scale, microalgae production is performed in a broad range of systems. In fact, cultures can be carried out in simple Erlenmeyer flasks, mechanically agitated reactors, or in flat panel photobioreactors (Figure 4).



Figure 5. Examples of lab-scale production of microalgae: mechanically agitated reactors, flat panel photobioreactors, and Erlenmeyer flasks. Based on Enzing et al., (2014).

With all this in mind, several factors influence the growth of microalgae and cyanobacteria, including light, temperature, pH, concentration of CO_2 and O_2 , nutrient concentration, salinity, mixing, and dilution rate. Therefore, these factors should always be into consideration when cultivating microalgae and cyanobacteria. In fact, photoperiod and light intensity are two of the most important factors on regulation and limitation of mixotrophic and autotrophic regimes and can be adjusted accordingly to the culture (Mata et al., 2010). A study showed that high (18h) or low periods (6h) of of daily light was important for cell growth in batch cultures of the cyanobacterium *Arthrospira maxima* and the microalgae *Chlorella vulgaris*, *Isochrysis galbana*, and *Nannochloropsis gaditana* (Sánchez-Bayo et al., 2020). Moreover, cyanobacteria like *Synechocystis* sp. and *Synechococcus* sp. are very highly light tolerant, showing maximum specific growth rates of 0.108 h^{-1} and 1.4 d^{-1} at an average irradiance of $930 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and 200 to $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$, respectively, without any photoinhibition (Singh & Singh, 2015). It is important to note that light availability does not depend exclusively on the light irradiation shedded upon microalgae's culture surface but also on the biomass density and photobioreactor configuration (Muñoz et al., 2015).

Synechocystis sp. is a unicellular non-nitrogen fixing cyanobacterium that is found in freshwater environments. It belongs to the Domain of Bacteria and the family of Merismopediaceae and exhibits versatile carbon metabolisms, being capable of growing in different conditions, showing high adaptability (Yu et al., 2013). Besides this, it was the first photoautotrophic to have a complete genome sequence,

having specific databases to thoroughly provide information about the regulation of gene expression of this organism. For this reason, *Synechocystis sp.* has become one of the most popular cyanobacterial models to study (Riediger et al., 2021).

1.3.2.2. Biotechnological applications and products of interest

In the last two decades, microalgae and cyanobacteria have been intensively studied due to its great potential in CO₂ mitigation. Microalgae-based approaches have been successfully implemented for this purpose because of its high photosynthetic efficiency, high ability of sequestering low-concentration CO₂, faster sequestration rate than higher plants, and not competing with crops for arable land (Zhou et al., 2017). Likewise, the capability of growing in many different environments — freshwater and marine water or even under extreme levels of pH and salinity – and widespread existence across the globe shows an immense adaptability and potential (Radmer, 2009). In addition, they present great potential to be applied in numerous biotechnological fields and tend to have a plethora of commercial applications. The lipids extracted from microalgae and cyanobacteria have been receiving a lot of attention due to its potential to generate raw materials involved in the production of third generation biofuels. Furthermore, they contain multiple high-value compounds like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), pigments (e.g., carotenoids, phycobiliproteins), vitamins (biotin, vitamin C, and vitamin E), biochemically stable isotopes, and other metabolites with pharmacological activities (e.g., antitumoral, antibacterial, anti-cholesterol, antifungal) – that are relevant for the Food and Feed industries, for instance, as food supplementation, or the pharmaceutical and cosmetics sector (Abed et al., 2009; Converti et al., 2009; Morais Junior et al., 2020). In the last few years, bioplastics obtained through algae biomass have increased in popularity due to their easy applicability and sustainability, as well as being certified to use in industrial packaging, catering products, gardening, and medical hygiene (Morais Junior et al., 2020). Moreover, with an expected increase of the world's population, the search for new and sustainable sources of food and energy is imperative. Cyanobacteria and microalgae arise as a good alternative to this problematic, as they provide a great variability of nutrients and precursors of biofuel production. In fact, algae can represent up to 18% of the protein sources by 2050 and it is estimated a market for the global algae protein of a 1 billion € by 2026. Carotenoids market has also been spreading, with a total value of approximately 1.5 billion € in 2019 (Hachicha et al., 2022).

1.3.3. Co-cultures of methanotrophic bacteria and microalgae/cyanobacteria

The co-cultivation of microalgae/cyanobacteria and aerobic methanotrophs can promote a meaningful synergistic interaction that have many advantages for the consortium itself and for the environment. Methanotrophic bacteria use CH₄ as source of carbon and O₂ for oxidation of this compound, whereas CO₂ is released. Microalgae/cyanobacteria use the carbon source released by the bacteria (CO₂) to produce O₂ that will be necessary for the metabolism of methanotrophs. One of the benefits of the co-culture relies on the fact that the exchange of *in situ* produced gases reduces mass transfer resistance, increasing the availability of these gases for each organism. Additionally, the consumption of O₂ and CO₂ diminishes the inhibition of photoautotrophs and methanotrophs, respectively (Badr et al., 2019).

Co-cultures of these organisms have been widely explored as a highly efficient way to recover energy from biogas streams and capture carbon from CH₄ and CO₂ to liberate O₂. This natural microbial communities are very common in nature and constitute a major sink of both GHGs while allowing for high-value biomass production (Badr et al., 2019). They can be cultured together, providing a robust synergistic interaction where possible metabolic links can be formed to promote or inhibit growth. Alternatively, the species can be cultured separately or sequentially (photoautotroph then methanotroph) offering flexibility. Several studies have been conducted using different concentrations of CH₄ and CO₂ for different purposes (i.e., CH₄ and CO₂ uptake, growth rate evaluation, assessment of metabolites produced) (Hill et al., 2017; Ruiz-Ruiz et al., 2020; Van Der Ha et al., 2012). Table 2 summarizes some of these examples as well as the conditions applied.

Table 2. Co-culture conditions using different species of methanotrophic bacteria and microalgae/cyanobacteria

Co-culture species	Headspace Conditions	Other conditions	Growth	Ref.
<i>Scenedesmus obtusiusculus</i> AT-UAM microalgae (GM) and alkaliphilic methanotrophic bacteria	4 % and 8 % CH ₄	Bottles: 125 mL; Media: MSM and BG-11 (50% v/v); pH: 9.15; Pressure: 1 bar; Light: 115 μmol m ⁻² s ⁻¹ ; GM/AMB Ratios: 1:1, 1:3; 3:1, 4:1, 5:1.	4 % CH₄ (GM/AMB): 1:1=31 %/69 %; 1:3=39 %/61 %; 3:1=27 %/73 %; 4:1=9 %/91 %; 5:1=2 %/98 %; 8 % CH₄ (GM/AMB): 1:1: 30.6 %/69.4 %; 1:3: 57.9 %/37.3 %;	Ruiz-Ruiz et al. (2020)

(AMB) consortium			3:1: 1.96 %/96.7 %; 4:1: 8 %/92 %; 5:1: 8 %/92 %;	
<i>Synechococcus</i> 7002 and <i>M.</i> <i>alcaliphilum</i> 20z	97 % CH ₄ + 3 % CO ₂	Continuous cultivation; T: 30° C; pH 8; Light: 250 μmol m ⁻² s ⁻¹ ; Pressure: 1 bar .	μ _{max} = 0.050 ± 0.005 h ⁻¹	Hill et al. (2017)
<i>Scenedesmus</i> <i>sp.</i> and <i>Methylocystis</i> <i>parvus</i>	Synthetic biogas	CSTR reactor: 3L; Pressure: 1 bar; Light: 60 μmol PAR m ⁻² s ⁻¹ pH 8.8 and pH 7; Media: dNMS; T: 20 ° C.	N.d.	(Van Der Ha et al. 2012)
<i>Chlorella</i> <i>sorokiniana</i> (Green microalgae GM) + <i>Methylococcus</i> <i>capsulatus</i> (Bath)(MB)	70% CH ₄ + 30% CO ₂	Bottles: 250mL Light: 200 μmol m ⁻² s ⁻¹ T= 37°C GM/MB Ratio: 3:1; Media: diluted AD effluent media (tap water (TW); secondary CLE (CLE); standard AMS medium without NH ₃ -N and PO ₄ ³⁻ -P (AMS)).	TW = 2.6 g/L; CLE = 3 g/L; AMS = 2.5 g/L.	Roberts et al. (2020)
<i>Chlorella</i> <i>sorokiniana</i> + <i>Methylococcus</i> <i>capsulatus</i>	60 % CH ₄ + 40% CO ₂	Bottles: 600 mL; Batch experiments; T: 37 °C; Media from an industrial wastewater; pH 7; Light: 2700 μmol m ⁻² s ⁻¹ .	μ _{max} = 0.21d ⁻¹	(Rasouli et al. 2018)

Nd. = not defined.

1.3.4. Potential role of microalgae and cyanobacteria on CH₄ consumption

As stated in section 1.1.4., many evidences suggest that microalgae/cyanobacteria are closely related to the production of high CH₄ concentrations (Beverdors et al., 2010; Gomez-Garcia et al., 2011; Tang et al., 2014). Nevertheless, Beverdors et al. (2010) hypothesized that CH₄ present in marine environments could be oxidized to CO₂ for an additional energy gain or could be used as biosynthetic precursors for cell growth of these microorganisms.

Evidence that supports this theory relies on the fact that many cyanobacteria and microalgae have been shown to be great phycoremediators in wastewater. Recently, these photosynthetic microorganisms showed efficient degradation of hydrocarbons (lowest: 24%/ highest: 100%) by using these compounds as a source of carbon for their growth (Das & Deka, 2019; Dell' Anno et al., 2021; Tomar & Jajoo, 2021). It is important to note that CH₄ is the elementary member of a series of hydrocarbons (alkanes). Several authors indicate that microalgae and cyanobacteria can degrade a wide range of hydrocarbons (e.g. alkanes, aromatic compounds, cycloalkanes); however, different strains have different affinity levels towards degradation and distinct potential for bioremediation (Hammed et al., 2016; Sample et al., 1999). In fact, *Scenedesmus obliquus* showed a higher preference for polycyclic hydrocarbons than n-alkanes, as opposed to *Nitzschia linearis* that showed great affinity towards the degradation of n-alkanes (Ibrahim & Gamila, 2004). Other studies indicate that *Nitzschia* sp. has higher degradation and accumulation abilities for fluoranthene (PAH) than *Skeletonema* sp., but poorer performances regarding phenanthrene (PAH) (Hong et al., 2008). In addition, *Chlorella vulgaris* and *Spirulina* sp. are also species known to efficiently degrade hydrocarbons with reductions up to 75% (Blanco-Vieites et al., 2022). Regarding the enzymatic pathway used in these circumstances, Luo et al. (2014) showed that PAHs (polycyclic aromatic hydrocarbons) were absorbed by *S. capricornutum* and *Chlorella* sp. cells and subsequently broken down through enzymatic activities, including o-diphenol oxidase, cytochrome P450, and peroxidase. Though, some studies show that these pollutants aren't always converted into beneficial chemicals, especially in the case of PHAs, as the adsorption of such compounds on cell wall's active groups by electrostatic attraction and complexation, as well as bioaccumulation of pollutants inside the cells, can also occur (Touliabah et al., 2022). Furthermore, it could be theorized that photosynthetic organisms may be able to use the shortest and simplest existing hydrocarbon, CH₄, as a source of carbon, since Ibrahim & Gamila (2004) showed that efficient degradation (68,9%) can happen in shorter chains of hydrocarbons (C10).

Cyanobacteria also constitute an ancient bacterial phylum that was responsible for the oxygenation of the atmosphere and oceans during the Great Oxidation Event. Nisbet & Fowler, (2011) suggested that in early/mid-Archaeon, the atmosphere was anoxic and the concentration of CO₂ and CH₄ was high, hinting at the presence of methanogenic archaea. Around 2.9 billion years ago, cyanobacteria started to appear, and the Great Oxidation Event took place, releasing O₂ and diminishing the concentration of CO₂ and CH₄ in the atmosphere. Because of this, the scientific community theorizes that cyanobacteria could have had an important role in diminishing CH₄ concentration (Demoulin et al., 2019; Hamilton et al., 2018; Nisbet & Fowler, 2011).

Moreover, microalgae and cyanobacteria have shown to have high tolerance for CH₄ (Nagarajan et al., 2019). In fact, when cultivated in biogas, these organisms show efficient removals of CO₂ in the presence of CH₄ (Choix et al., 2017). For instance, *Scenedesmus obliquus U169* was shown to have higher CO₂ fixation rates when grown in 25% CO₂ and 75% CH₄ compared to 25% CO₂ and air. Additionally, *Nannochloropsis gaditana* presented good resistance levels to extremely high levels of CH₄, without any significant changes in biomass production and growth rate (Rivas & Jeison, 2014). This demonstrates the low toxicity to CH₄ that these photosynthetic organisms have.

Considering all this evidence and potential, cyanobacteria/microalgae arise as a good alternative to overcome several disadvantages of CH₄ mitigation, presenting a new and innovative strategy as well.

2. Aims

The main goal of this project was to develop an innovative and sustainable CH₄ mitigation system by identifying photosynthetic microorganisms that can metabolize CH₄ in pressurized systems, thus reducing its greenhouse effect.

The first specific aim of this work was to understand if cyanobacteria could use CH₄ as a carbon source for growth or at least withstand high concentrations of this GHG.

The second aim was to promote high consumption rates of GHGs in co-cultures of cyanobacteria and methanotrophic bacteria, particularly CH₄ and CO₂. The correlation between the gas consumption and growth of each microorganism, nitrate and orthophosphate consumption, and biomass protein content was also evaluated.

3. Materials and Methods

3.1. Strains

Synechocystis sp. LEGE 07211 was kindly provided by the LEGE culture collection, from CIIMAR. This cyanobacteria species was isolated from a wastewater treatment plant at Febros river, Vila Nova de Gaia. The methanotrophic bacteria was obtained through an enrichment with high relative abundance of *Methylococcus* sp. collected from a sludge sourced from a wastewater treatment station located at the northern region of Portugal by the Environmental Biotechnology Laboratory of the Centre of Biological Engineering of University of Minho that was incubated with high concentrations of CO₂, CH₄, and O₂. During the present work this enrichment will be called *Methylococcus* sp.

3.2. Culture Media

The Z8 medium was used for the maintenance and experiments of the selected cyanobacteria species and in the co-culture assays. For Z8 medium preparation, firstly, four stock solutions were prepared: Solution A (46.7 g/L NaNO₃, 5.9 g/L Ca (NO₃)₂·4H₂O, 2.5 g/L MgSO₄·7H₂O); Solution B (3.1 g/L K₂HPO₄, 2.1 g/L Na₂CO₃); Micronutrients solution (0.33 g/L Na₂WO₄·2H₂O, 0.88 g/L (NH₄)₆Mo₇O₂₄·4.2H₂O, 1.2 g/L KBr, 0.83 g/L KI, 2.87 g/L ZnSO₄·7H₂O, 1.55 g/L Cd(NO₃)₂·4H₂O, 1.46 g/L Co(NO₃)₂·6H₂O, 1.25 g/L CuSO₄·5H₂O, 1.98 g/L NiSO₄(NH₄)₂SO₄·6H₂O, 0.41 g/L Cr(NO₃)₃·9H₂O, 0.089 g/L V₂O₅, 9.48 g/L AlK(SO₄)₂·12 H₂O, 3.1 g/L H₃BO₃, 2.23 g/L MnSO₄·4H₂O) and Fe-EDTA solution: (2.8 g/100 mL FeCl₃·6H₂O; HCL (0.1 N), 3.9 g/100mL EDTA, NaOH (0.1N)). Finally, 10mL of the solutions A, B and Fe-EDTA solution, as well as 1 mL of the micronutrient's solution, were added to 969 mL of distilled H₂O in order to have the Z8 medium completed.

The dNMS medium was used for the maintenance and some experiments with *Methylococcus* sp. This medium was prepared following the protocol of (BCCM, 2023). It is important to note that this species was maintained in pressurized bottles at 2 bar sealed with a septum and aluminium caps. The gas mixture used was 40 % CH₄ + 40 % CO₂ + 2 % N₂ with a ratio of workspace: headspace of 1:10.

All materials and media used in this work were previously autoclaved for 40 min at 121°C and the manipulation of all cultures and experiments was performed in a laminar flow chamber/by the flame with sterilized material.

3.2.1. *Methylococcus* sp. adaptation phase

The culture of this methanotrophic bacteria was given under specific culture conditions and it was slowly adapted during 30 days in order to be used in the experiments of this work, as demonstrated in Table 3.

Table 3. Adaptation of *Methylococcus* sp. culture conditions.

Culture conditions	Initial culture conditions	Adapted conditions
Medium	dNMS	Z8
Temperature	30° C	Room temperature
Light cycle	dark	12:12

3.3. *Synechocystis* sp. cultures in pressurized reactors

To evaluate the possible consumption of CH₄ by *Synechocystis* sp. LEGE 07211, different combinations of CO₂ and CH₄ concentrations - 0.5 % CO₂:0.5 % CH₄ – 1 % CO₂:1 % CH₄ - 2.5 % CO₂: 2.5 % CH₄ - were studied in a closed atmosphere, being 2 % CO₂ the positive control. Additionally, it was also performed a set of experiments where different concentrations of CH₄ in the atmosphere were tested: 1% CH₄, 2 % CH₄, 5 % CH₄ and 20 % CH₄.

These experiments were carried out using 70 mL serum bottles sealed with a butyl rubber septum and an aluminium capsule containing a final culture volume of 7 mL. The work volume/headspace ratio was therefore of 1:10. Serum bottles were previously washed with chloramine (10 g/L) to avoid any potential contamination and further dried out in a WTC Binder Heating Chamber at 105° C (TUTTLINGEN/GERMANY). Cells were cultivated, through a syringe, in autoclaved Z8 medium buffered with 70 mM of HEPES to maintain pH at 8.

The headspace of each bottle was filled with pressurized air, pure CO₂ and pure CH₄ in previously calculated volumes using Henry's law (Eq. 3. Section 3.5.), considering a final pressure of 2 bar. Finally, the bottles were autoclaved for 40 minutes at 121° C in autoclave bags.

The bottles were then placed vertically on VWR STD 5000 orbital shaker (USA) at room temperature under a light intensity of 30 µmol m⁻² s⁻¹ - using LED lighting systems - measured through a LI-191R Line Quantum Sensor, USA, under a 12:12 light/dark cycle.

Liquid and gas samples were taken every 4 days for 12 days. Liquid samples (200 µL) were retrieved next to a flame using a syringe of 1 mL and analysed through optical density (750nm) in a 96-well plate reader (Biotek Cytation 3 Cell Imaging Multi-Mode Reader (USA)). pH levels were also read using a Hanna Instruments Electrode HI330B (Romania). Gas samples were taken with a 1 mL syringe

for CH₄ (500 µL), CO₂ (200 µL) and O₂ (500 µL) evaluation. The headspace analysis was monitored by gas chromatography (GC) using a GC-2014 Shimadzu (Kyoto, Japan), fitted with a Porapak Q 80/100 mesh, a packed stainless-steel column (2 m × 1/8 inch, 2 mm), and a flame ionization detector (FID) and a Brucker scion 456-GC Chromatograph with the flame ionization detector TCD for CO₂ and O₂ assessment. All headspace gas were sampled using a pressure-lock syringe (Hamilton, Reno, Nevada, EUA).

3.4. Co-cultures of methanotrophs and cyanobacteria

3.4.1. Evaluation of different culture conditions on *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp.

Synechocystis sp. LEGE 07211 and *Methylococcus* sp. were tested under different conditions, as demonstrated in Table 4.

Table 4. Tested conditions for *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp.

a) <i>Synechocystis</i> sp. LEGE 07211 with 8% CO₂ (250 mM HEPES)	b) <i>Methylococcus</i> sp. w/ 40% CH₄ + 40 % CO₂ + 20 % N₂	c) <i>Methylococcus</i> sp. w/ 40% CH₄ + 40 % CO₂ + 20 % N₂ (pH 7)
Z8 medium with pH 7	dNMS with 250 mM HEPES (pH 7)	Z8 without HEPES
Z8 medium with pH 8	Z8 with 250 mM HEPES (pH 7)	Z8 with 30 mM HEPES
dNMS medium with pH 7	50 % (v/v) Z8+ 50 % (v/v) dNMS medium with 250 mM HEPES (pH 7)	Z8 with 60 mM HEPES
dNMS medium with pH 8	dNMS without HEPES (pH 6)	Z8 with 125 mM HEPES
50% (v/v) Z8+dNMS medium with pH 8	50 % (v/v) Z8+ 50 % (v/v) dNMS medium without HEPES (pH 6)	-
-	Z8 without HEPES (pH 6)	-

Three different preliminary experiments were performed under pressurized atmosphere of 2 bar and a work volume/headspace ratio of 1:10. The pH was adjusted using NaOH 1M and HCL 1 M in all experiments. All cultures were placed horizontally to increase surface area on an orbital shaker at room temperature under light intensity of 20 µmol m⁻² s⁻¹ and light-dark cycle of 12:12. The methodology for sampling and analysis was the same as mentioned in section 3.3., except in experiment c) where only absorbance was evaluated. Samples were taken every 2 days 10 days in the case of *Synechocystis* sp.

LEGE 07211 (a) and *Methylococcus* sp. (c) cultures, and every day, twice a day during 4 days, for *Methylococcus* sp. growths in experiment b.

3.4.2. Combined growth of microorganisms

Based on a Design of Experiments (DOE) methodology, using Protimiza Experimental Design software (<http://experimental-design.protimiza.com.br>), different gas compositions and ratios of *Synechocystis* sp. and *Methylococcus* sp. were tested in order to evaluate biogas consumption. Therefore, a CCD (Central Composite Design) was used to assess gas consumption, in particular of CH₄ and CO₂, and relate this to the biomass growth of each microorganism in co-culture. This method was applied using 4 independent variables: O₂ concentration (x₁), CH₄ concentration (x₂), CO₂ concentration (x₃), and ratio of *Synechocystis* sp. LEGE 07211 (x₄) (Table 5).

Table 5. Identification of the variables (4) tested in CCD and the corresponding coded levels.

Code	Variables	Unit	Levels		
			-1	0	1
x ₁	O ₂ concentration	%	5	10	15
x ₂	CH ₄ concentration	%	5	25	45
x ₃	CO ₂ concentration	%	5	12.5	20
x ₄	Ratio of <i>Synechocystis</i> sp. LEGE 07211	%	25	50	75

Considering the range of values tested, an experimental design was planned with a total of 19 runs, consisting of 16 runs with different conditions and 3 replicates at level 0 – as central points (Table 6.)

Table 6. Growth conditions tested in the experimental design.

Experiments	O ₂ Concentration (%)	CH ₄ concentration (%)	CO ₂ concentration (%)	Ratio of <i>Synechocystis</i> sp. LEGE 07211 (%)
	X ₁	X ₂	X ₃	X ₄
1.	5	5	5	25
2.	15	5	5	25
3.	5	45	5	25
4.	15	45	5	25
5.	5	5	20	25
6.	15	5	20	25
7.	5	45	20	25
8.	15	45	20	25
9.	5	5	5	75
10.	15	5	5	75
11.	5	45	5	75
12.	15	45	5	75
13.	5	5	20	75
14.	15	5	20	75
15.	5	45	20	75
16.	15	45	20	75
17.	10	25	12.5	50
18.	10	25	12.5	50
19.	10	25	12.5	50

As the aim of this set of experiments was to evaluate gas consumption and its impact on microorganisms' growth, 8 response variables were established (Table 7).

Table 7. Response variables studied through the CCD.

Code	Unite	Variables of response
y ₁	Counts/mL	Productivity <i>Synechocystis</i> sp. LEGE 07211
y ₂	Counts/mL	Productivity <i>Methylococcus</i> sp.
y ₃	%	CO ₂ balance
y ₄	%	CH ₄ balance
y ₅	%	O ₂ balance
y ₆	%	Removal of nitrates
y ₇	%	Removal of phosphates
y ₈	mg/L	Total nitrogen

These experiments were carried out in 240 mL serum bottles containing a final culture volume of 24 mL, considering a work volume/headspace ratio of 1:10. The serum bottles were previously washed with chloramine (10 g/L). Autoclaved Z8 medium buffered with 30 mM of HEPES (to maintain pH at 7) was added and bottles were closed with a butyl rubber septum and an aluminium capsule. The headspace of these bottles was washed by applying 8 cycles of sequential headspace vacuum removal/replacement with N₂, and then filled, through 60 mL syringe, with different concentrations of pure CH₄, CO₂ and O₂, according to each condition of the experimental design. As performed in the previous assays – sections 3.3 and 3.4.1 - the absolute pressure of the bottles was maintained at 2 bar.

Regarding the inoculation procedure, cells were washed 3 times with 0.85% NaCl, pH 7, previously autoclaved. This isotonic solution was used in order to remove the nutrients from the inoculum. Each culture of microorganisms was homogenized and centrifuged using Thermo Scientific Heraeus Multifuge X3R (Germany) at 15000g and 21° C for 10 min. Afterwards the correct dilution, according to each ratio tested (25 %; 50 % and 75 %) was done and introduced in each bottle in order to have a final OD (750nm) of the co-culture of approximately 0.15. Finally, all cultures were placed horizontally – to increase surface area – on an orbital shaker at room temperature under light intensity of 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and light-dark cycle of 12:12.

Liquid samples (250 μL) were taken, under sterile conditions, every 2 days for pH monitoring. Additionally, flow cytometry analysis was performed using a Cytoflex Beckman Coulter. Gas samples were also taken and analysed as mentioned in section 3.3.

When the stationary phase of the co-culture was reached by both microorganisms, cultures were centrifuged using a Thermo Scientific Heraeus Multifuge X3R at 15000g, and -4° C for 10 min. The resulting supernatant and pellet were separated and frozen at -4 °C. The supernatant was later analysed in order to evaluate the concentration of nitrates, orthophosphates and total nitrogen (section 3.4.3). On the other hand, the pellet was analysed to determine the total nitrogen content (section 3.4.3.).

3.4.3. Nitrate, orthophosphate, and protein quantification

The amount of nitrates, orthophosphate, and total nitrogen was determined spectrophotometrically in the beginning and the end of each assay using the cuvette test kits LCK 339, 350, and 338 (Hach-Lange GmbH, Düsseldorf, Germany), respectively, according to the manufacturer's instructions.

3.5. Data analysis

Using the Ideal Gas Law (Eq. 1) the number of mols (n) of CO₂, CH₄ and O₂ present in the headspace and workspace of each vial was determined. Afterwards, the concentration of each gas was calculated using the volume of workspace and the number of mols (n) in the headspace.

$$P.V = n.R.T \quad (3)$$

where P , V , n , R and T represent pressure (atm), volume (L), number of mols (mol), ideal gas constant (0.08205 L.atm)/(K.mol), and temperature (K), respectively.

The specific consumption rate of each monitored gas (CO₂, CH₄ and O₂)– Eq.4 – was estimated as follows:

$$C_{gas} = \frac{C_2 - C_1}{t_2 - t_1} \quad (4)$$

where c_1 and c_2 are gas concentration (mmol/L) at time 1 (t_1) and time 2 (t_2), respectively.

Maximum specific growth rate (μ_{max}) – Eq 5 - was estimated for *Synechocystis* sp. and *Methylococcus* sp. using the equation:

$$\mu_{max} = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad (5)$$

where x_1 and x_2 are counts/mL at time 1 (t_1) and time 2 (t_2) in the exponential growth phase, respectively.

Biomass productivity (P_x) – Eq 6 - was estimated for *Synechocystis* sp. and *Methylococcus* sp. using the equation:

$$P_x = \frac{x_2 - x_1}{t_2 - t_1} \quad (6)$$

where x_1 and x_2 are counts/mL at time 1 (t_1) and time 2 (t_2).

Balance of each gas (CO₂, CH₄, and O₂) – was estimated as follows:

$$Balance_{gas} = \frac{C_2 - C_1}{C_1} \times 100 \quad (7)$$

where c_1 and c_2 are the gas concentrations (mmol/L) at the beginning and the end of each assay, respectively.

Nitrate and phosphate consumption was calculated using the equation:

$$Consumption_{Nitrate\ and\ Phosphate} = \frac{C_2 - C_1}{C_1} \times 100 \quad (8)$$

where c_1 and c_2 are the concentration of nitrates and phosphates (mg/L) at the beginning and the end of each assay, respectively.

Total nitrogen was calculated using the equation:

$$Total\ nitrogen = C_2 - C_1 \quad (9)$$

where c_1 and c_2 are the total nitrogen (mg/L) at the beginning and the end of each assay, respectively.

3.6. Statical analysis

The significant variables in the CCD design were selected using a 90% confidence level ($p < 0.10$) and the quality of the fitted model was statistically evaluated by analysis of variance (ANOVA) and coefficient determination (R^2). DCC analysis was performed with the online software Protimiza Experimental Design (<http://experimentaldesign.protimiza.com.br/>).

4. Results and discussion

4.1. Effect of different concentrations of CH₄ and CO₂ on *Synechocystis* sp. LEGE 07211

In previous studies of our research group, it was determined that *Synechocystis* sp. LEGE 07211 could grow under closed pressurized atmosphere with 2% CO₂ (2 bar) and 70mM of HEPES buffer. With that in mind, it was possible to evaluate the effect that CH₄ could have on these photosynthetic organisms using pressurized systems. Firstly, different concentrations of CO₂ + CH₄ (0.5 %, 1 %, and 2.5 %) at a ratio 1:1 were tested. Lower concentrations were used in order to understand if the use of CH₄ as carbon source, by *Synechocystis* would be possible after CO₂ depletion or if the presence of CH₄ would trigger cyanobacterial metabolism resulting in increased growth.

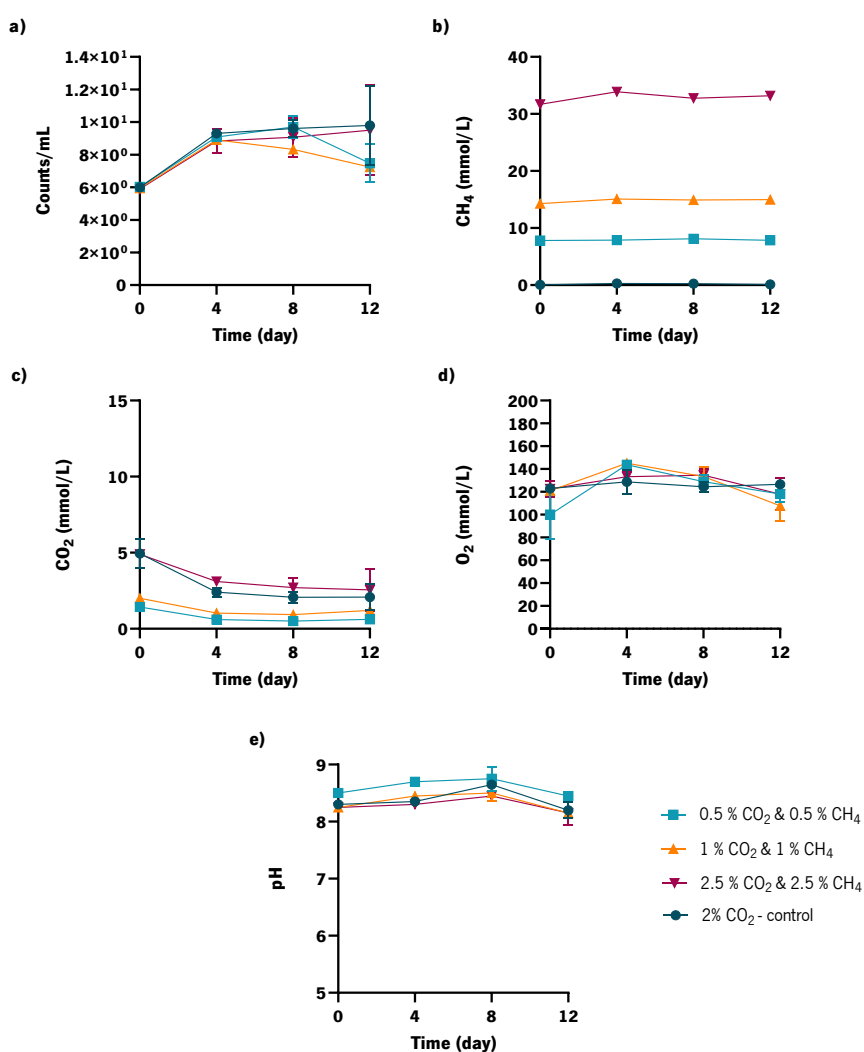


Figure 6. Effect of different concentrations of CO₂ + CH₄ on *Synechocystis* sp. LEGE 07211 cultures a) growth, b) CH₄ concentration (mmol/L), c) CO₂ concentration (mmol/L), d) O₂ concentration (mmol/L), and e) pH variation. Error bars indicate the minimum and maximum values observed (n=2). ■ – 0.5 % CO₂ & 0.5 % CH₄; ▲ – 1 % CO₂ & 1 % CH₄; ▼ – 2.5 % CO₂ & 2.5 % CH₄; ● – 2 % CO₂.

In Figure 6, very similar biomass concentration of *Synechocystis* sp. LEGE 07211 were obtained in all conditions. In fact, 2.5 % CH₄ + 2.5 % CO₂ started with 5.89 ± 0.03 counts/mL, reaching the highest value of 9.50 ± 1.96 counts/mL on day 12. Moreover, cultures grown under 0.5 % CH₄ + 0.5 % CO₂ and 1 % CH₄ + 1 % CO₂ reached 9.71 ± 0.5 and 8.90 ± 0.06 counts/mL on day 8 and day 4, respectively. Each one of these growths are quite similar to the control (2% CO₂), which reached the highest value of 9.80 ± 1.73 counts/mL on day 12.

When analysing the pH values, it can be observed an increase until day 8 in all conditions, with a subsequent sharp decrease. Mavromatis et al. (2012) stated that when a reduction in photosynthetic activity is observed, it can result in a decrease of pH. Additionally, this is concurrent with a decrease in optical density, suggesting that this observation may stem from cyanobacterial death.

Regarding CO₂ concentrations, the conditions with less CO₂ (0.5 % CH₄ + 0.5 % CO₂ and 1 % CH₄ + 1 % CO₂) appear to suffer an early decrease in biomass growth on day 8 and 4, respectively. This decline could be explained by the depletion of this gas observed in these days. Moreover, when observing the control and 2.5 % CH₄ + 2.5 % CO₂, it can be noted that they have very similar concentrations. Meier et al. (2015) has shown that in the presence of CH₄, the removal effect of CO₂ increased by 99.3 %, however, this study used significantly higher concentrations of CO₂ (30 %) and CH₄ (40 % - 50 %) than those tested in the present experiment. Thus, it could be possible that this phenomenon does not occur at such low concentrations. Nevertheless, this hypothesis should be further evaluated.

Table 8. Consumption rates of CO₂, CH₄, and O₂ of *Synechocystis* LEGE 07112 using different conditions of CO₂ and CH₄. Negative values represent gas consumption, while positive values represent its production. The values are the mean of two different assays (n = 2) with the deviation representing the maximum and minimum values obtained.

Condition	Rate of CO ₂ (mmol.L/day)	Rate of CH ₄ (mmol.L/day)	Rate of O ₂ (mmol.L/day)
0.5 % CO₂ + 0.5 % CH₄	- 0.07 ± 0.01	nd	1.50 ± 0.88
1 % CO₂ + 1 % CH₄	- 0.07 ± 0.01	0.06 ± 0.02	-1.10 ± 0.97
2.5 % CO₂ + 2.5 % CH₄	- 0.20 ± 0.09	0.13 ± 0.01	- 0.39 ± 1.24
2 % CO₂ – control	- 0.24 ± 0.01	nd	0.30 ± 0.11

nd - not detected

Focusing only on CH₄ concentrations, these kept stable throughout the entire experiment, apart from the assays with 1 % CO₂ + 1 % CH₄ and 2.5 % CO₂ + 2.5 % CH₄ that seem to have a residual production of this gas – Table 8. It is important to note that this rate is very residual. Additionally, it is possible to observe that the control (2% CO₂) does not show any production of this gas. The production of CH₄ in cyanobacteria has been reported before in previous studies, associating this with photosynthesis process

(Bižić et al., 2020; Günthel et al., 2020). In these studies, the authors state that CH₄ production happens at the same time as O₂ increases. However, the pathways involved in this process are not known yet and need to be further investigated. Contrarily to this hypothesis, the results presented in figure 6 and table 8, show a residual reduction of O₂ concentration, to which contradicts this theory. Moreover, this particular species is not described as CH₄ producer. Nevertheless, since this assay was a preliminary assay tested in duplicate, no conclusions could be retrieved statistically. Therefore, a triplicate assay of these conditions should be performed in the future in order to understand if these production of CH₄ and decrease in O₂ concentration is significant.

These experiments suggest no interference of *Synechocystis* sp. LEGE 07211 in the consumption of CH₄ under the tested conditions. Only possible production was observed, although it still needs further investigation. Moreover, the growth seems to be related to the consumption of CO₂ in all conditions, but the interference of CH₄ still needs to be verified. Altogether, the use of low concentrations of CH₄ and CO₂ might have also contributed to the lack of robust conclusions regarding the effect of such gases on both biomass growth and gas consumption rates.

4.2. Effect of different concentrations of CH₄ on *Synechocystis* sp. LEGE 07211

Following the of section 4.1., an experiment using only CH₄ as a carbon source was performed, in order to understand if it would have any interference. In this assay a wider range of concentrations was tested, to unveil any potential impacts of the amount of this GHG in the atmosphere on cyanobacteria.

When analysing Figure 7, it can be noted that no growth was observed in all conditions; however, no decline was seen as well. Cyanobacteria and microalgae have shown to have high tolerance to CH₄, which could explain why no decrease in biomass concentration happened either when the culture was firstly inoculated or within the first 4 days. In fact, Meier et al. (2015) has shown that microalgae and cyanobacteria have a tolerance of 100% to CH₄. Nevertheless, this does not explain why the biomass concentration did not decrease for 12 days with no additional carbon source in the cultures. A possible justification for this could be related to the use of carbohydrate reserves that were present in the cells. These compounds, such as glycogen, are present frequently in cyanobacteria and microalgae when a nutrient is absent, which commonly occurs in the stationary phase. This compound allows the cells to cope with transient starvation conditions in order to survive. However, Gründel et al. (2012) mention that this mainly happens when a macronutrient, as the case of phosphorus or nitrogen, is absent or limiting

but a carbon source is present. Since there was no growth, as observed in Figure 7, the nutrients in the medium should be present, however it could be possible that if the carbohydrates were in fact used, they could come from the cell growth in pre-inoculum. Nevertheless, no studies were found regarding this process occurring in early stages of growth, which might have happened in the present work.

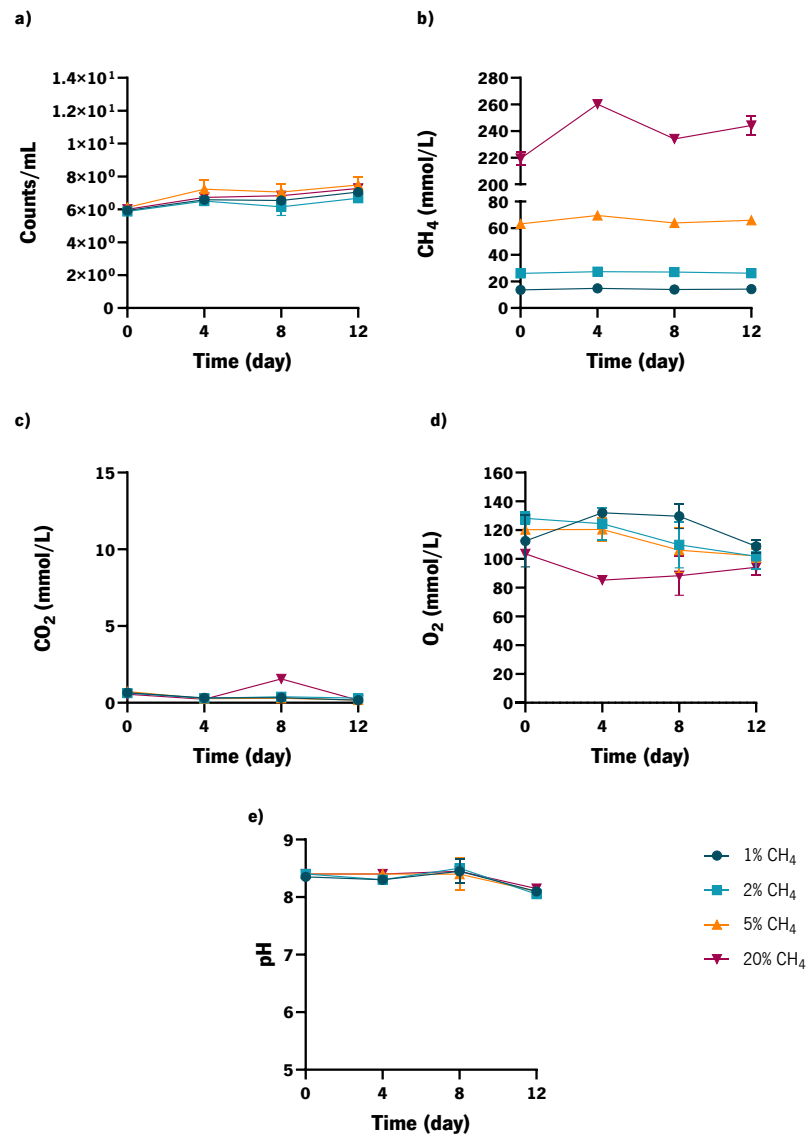


Figure 7. Effect of *Synechocystis* sp. LEGE 07211 cultivation under different concentrations of CH₄ on a) growth (counts/mL), b) CH₄ concentration (mmol/L), c) CO₂ concentration (mmol/L), d) O₂ concentration (mmol/L) and pH variation. Error bars indicate minimum and maximum values observed (n=2). ● – 1% CH₄; ■ – 2% CH₄; ▲ – 5% CH₄; ▼ – 20% CH₄.

When following the variation of O₂ throughout the time, a consumption is visible in all conditions (Table 9.). One possible explanation could be the cell's photorespiration, which is a light dependent process where O₂ is consumed, and CO₂ is produced that occurs simultaneously with photosynthetic CO₂ uptake and O₂ release (photosynthesis). Photorespiration happens during the fixation of CO₂. Under normal conditions of CO₂, RuBisCo fixes CO₂ and generates 3-PGA, that will then be used for biomass growth.

When the ratio of CO₂: O₂ is lower (lower concentrations of CO₂ and higher concentrations of O₂), photorespiration takes place, and RuBisCo uses O₂ to produce 2-PGA. Since this compound isn't useful for the cell, many different reactions happen using extra energy to convert it to 3-PGA. This process happens in 3 different organelles of the cell: mitochondria, chloroplast, and peroxisome – and releases CO₂ in the process. After this, the CO₂, instead of being wasted, is used efficiently during photosynthesis, since both processes occur at the same time. Since it requires extra energy, photorespiration is used mainly for cell maintenance under highly stress situations such as lack of CO₂ but high concentrations of O₂ (Hagemann & Bauwe, 2016). The conditions provided in this experiment could potentially drive this process to occur. This could be an explanation for the maintenance of cells and the decrease of O₂ observed. Moreover, since no concentrations of CO₂ were detected during the experiment, apart from a slight production of CO₂ in the condition in 20 % CH₄ on day 8, this could be explained by the photosynthesis occurring simultaneously to photorespiration.

A distinct production of CH₄ concentrations was denoted in the condition with 20 % CH₄, while a slighter increase was obtained under 5% CH₄, which can be observed in the CH₄ rate (Table 9). Subsequently, a repetition of this assay should also be done in triplicate in order to understand if this CH₄ production and consumption of O₂ is statistically significant. An extended assay could also be performed in order to understand whether the maintenance of the culture happens for longer periods of time under these conditions, or the decrease of growth happens after a certain period of time. Regarding pH values, a distinct decrease occurs only on day 8.

Table 9. Consumption rates of CO₂, CH₄, and O₂ of *Synechocystis* LEGE 07112 using different concentrations of CH₄. Negative values represent gas consumption, while positive values represent its production. The values are the mean of two different assays (n = 2) with the deviation representing the maximum and minimum values obtained

Condition	Rate of CO ₂ (mmol.L/day)	Rate of CH ₄ (mmol.L/day)	Rate of O ₂ (mmol.L/day)
1 % CH₄	nd	0.04 ± 0.01	- 0.31 ± 0.8
2 % CH₄	nd	0.02 ± 0	- 2.20 ± 0.26
5 % CH₄	nd	0.24 ± 0.11	- 1.52 ± 0.08
20 % CH₄	nd	2.06 ± 0.71	-0.78 ± 0.47

nd - not detected

These experiments suggest no consumption of CH₄ was observed. In fact, the opposite (production) was detected. However, it was possible to note that *Synechocystis* sp. LEGE 07211 has an incredible high tolerance to CH₄.

4.3. Evaluation of different culture conditions on *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp.

Since no consumption of CH₄ was observed under a pure culture of *Synechocystis* sp. LEGE 07211, a new and different strategy was set into place where a consortium of methanotrophic bacteria and *Synechocystis* sp. LEGE 07211 were tested in order to assess the consumption of CH₄ and CO₂ in co-culture.

4.3.1. *Synechocystis* sp. LEGE 07211 growth in different culture media

In order to combine both species in a powerful consortium, it was necessary to understand which conditions were the most favourable to each strain. The pH and the culture medium utilized appropriated for the co-culture of cyanobacteria and methanotrophic bacteria are two of the most important factors to have into consideration. In fact, Z8 medium is widely used for cyanobacteria and freshwater microalgae growth, while dNMS is a common medium for methanotrophic bacteria (Kwon et al., 2019; Lopes & Vasconcelos, 2011). For that reason, dNMS and Z8 media were tested at pH 7 and pH 8 for the growth of *Synechocystis* sp. LEGE 07211. Additionally, 50% (v/v) Z8+dNMS was also assessed at pH 8. All experiments were performed using HEPES 250 mM, based on previous assays of *Synechocystis* sp. LEGE 07211 carried out by our research group.

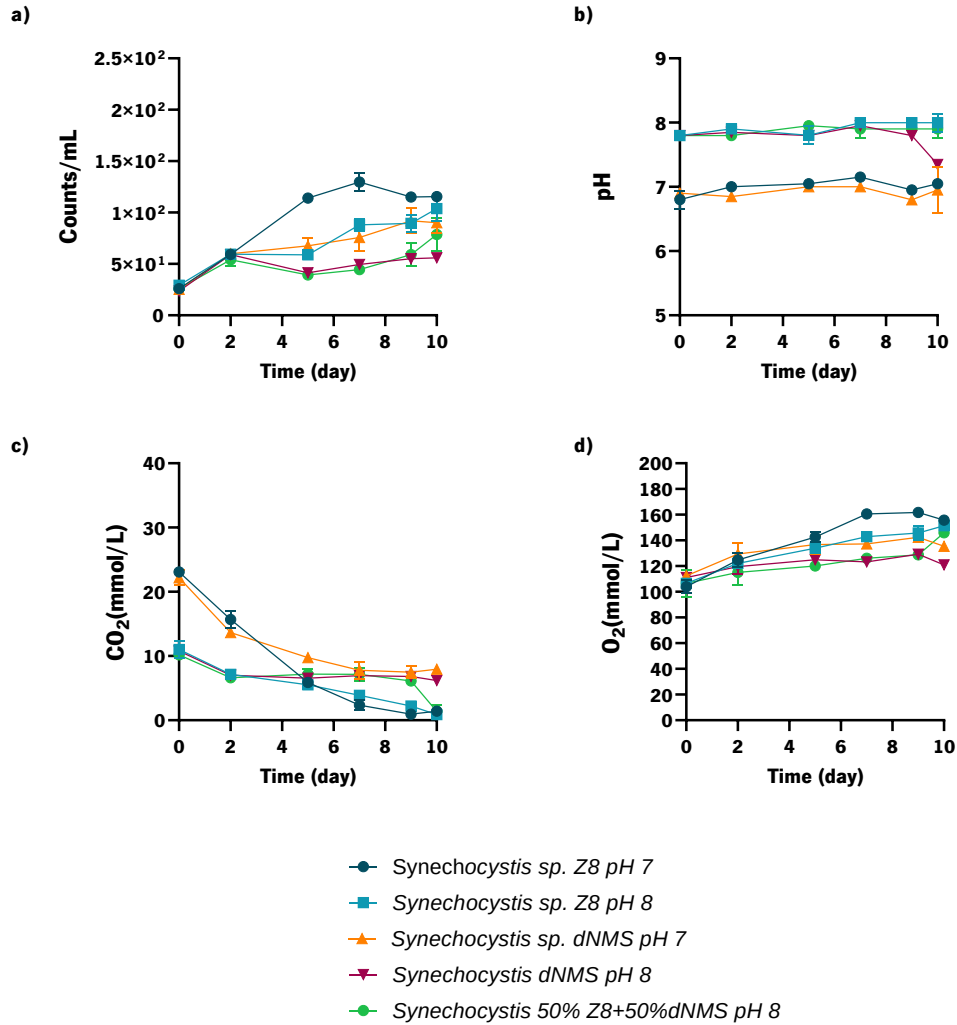


Figure 8. Effect of different growth media – (dNMS, Z8) with pH 7 and pH 8 and 50 % (v/v) DNMS+Z8 at pH 8 – on a) growth, b) pH variation, c) CO₂ variation (mmol/L), and d) O₂ variation (mmol/L) of *Synechocystis* sp. LEGE 07211 cultures. Error bars indicate minimum and maximum values observed (n=2). ● – *Synechocystis* sp. Z8 pH 7; ■ – *Synechocystis* sp. Z8 pH 8; ▲ – *Synechocystis* sp. dNMS pH 7; ▼ – *Synechocystis* sp. dNMS pH 8; ● – *Synechocystis* sp. 50 % (v/v) Z8 + 50 % (v/v) dNMS pH 8.

As demonstrated in Figure 8, there was growth in all conditions. However, there is a distinct increase on day 7 in Z8 medium at pH 7 (129.76 ± 6.3 counts/mL). This condition also presents the highest μ_{max} , productivity, and X_{max} , as displayed in Table 10. Such growth can be corroborated by Martínez et al. (2011), which mentions that, at lower pH values (pH 7), there is an increase in the biofixation of CO₂, thus increasing biomass production and the consumption rate of this gas. This study also demonstrates that *Synechocystis* sp. may prefer low concentrations of CO₂ in its culture medium. Therefore, pH 7 seems to be the best option for this species. Additionally, since there is a higher solubility of CO₂ in water at higher pH values, resulting in HCO₃⁻, this could explain why experiments with pH 8 started with lower CO₂ concentration than experiments with pH 7 (Zeebe & Wolf-Gladrow, 2001). Based

on data from Figure 8, it is also possible to note that Z8 medium at pH 7 appears to have a greater consumption rate when compared to others, as well as an expected higher production rate of O₂.

The growth observed in Figure 8 demonstrates that (v/v) 50% Z8+50% dNMS was very similar to dNMS pH 8 until day 9. However, on day 10, the first continued to grow, while dNMS pH 8 maintained its growth behaviour. In Table A1. it is possible to note that dNMS has a slight higher concentration in most macronutrients than Z8 medium, although there is a decrease of trace elements such as Co, Mn, Mo and Zn that could diminish the performance of this strain. In fact, Mn plays a key role on the molecular structure of the oxygen-evolving complex of PSII (photosystem II) and without them, significant decreases in O₂ evolution rates could be observed (Cuellar-Bermudez et al., 2015; Lamb & Hohmann-Marriott, 2017). This can be corroborated in O₂ concentrations. In condition with 50% (v/v) Z8 + 50% (v/v) dNMS (pH 8), O₂ concentrations continued to increase whilst in dNMS pH 8 a decreased was observed. Possibly due to this reason, higher global productivity is achieved in 50% (v/v) Z8 + 50% dNMS, when comparing it to dNMS pH 8 (Table 10.). Nevertheless, the μ_{max} of all conditions, except Z8 medium pH 7, is very similar. On the contrary different X_{max} were achieved in these conditions, being the lowest productivity and x_{max} achieved by the culture grown in dNMS medium and pH 8.

Table 10. Effect of different growth media and pH values on the maximum growth rate (μ_{max}), global biomass productivity (Productivity), maximum biomass concentration (X_{max}), consumption rate of CO₂, and production rate of O₂ of *Synechocystis* LEGE 07112. Negative values in CO₂ rate represent consumption, while positive values in rate O₂ represent production. The values are the mean of two different assays (n = 2) with the deviation representing the maximum and minimum values obtained.

Condition	μ_{max} (Day ⁻¹)	Productivity (Counts.mL/day)	X _{max} (Counts/mL)	Rate CO ₂ (mmol.L/day)	Rate O ₂ (mmol.L/day)
Z8 medium pH 7	0.16 ± 0.01	8.94 ± 0.01	129.76 ± 6.3	- 1.67± 0.05	3.99 ± 0.27
Z8 medium pH 8	0.07± 0.01	7.44 ± 0.87	103.79 ± 8.35	- 0.78 ± 0.07	3.45 ± 0.06
dNMS medium pH 7	0.06 ± 0.01	6.49 ± 0.69	91.97 ± 8.67	- 1.10 ± 0.07	1.77± 0.18
dNMS medium pH 8	0.06 ± 0.01	3.43 ± 0.46	56.01 ± 2.46	- 0.35 ± 0.03	0.76 ± 0.02
50% (v/v) Z8+dNMS medium pH 8	0.06 ± 0.01	5.28 ± 1.17	78.65 ± 11.23	- 0.68 ± 0.03	3.04 ± 0.75

The results of section 4.3.1. suggest that *Synechocystis* sp. adapts well at lower pH values (pH 7) and can grow in all media and pH levels tested, having higher growth rate in Z8 medium at pH 7. Moreover, in conditions with dNMS, O₂ production rate is reduced while more is CO₂ present in the culture medium at higher pH values which this particular species/strain may not prefer.

4.3.2. *Methylococcus* sp. growth using different culture conditions

4.3.2.1. *Methylococcus* sp. using different growth media

To understand if *Methylococcus* sp. would follow the same trend as *Synechocystis* sp. LEGE 07211 in terms of growth, a trial was carried out using the same 3 different media - dNMS, Z8, and 50 % (v/v) Z8 + 50 % (v/v) dNMS - at pH 6 (optimum pH for this specific strain).

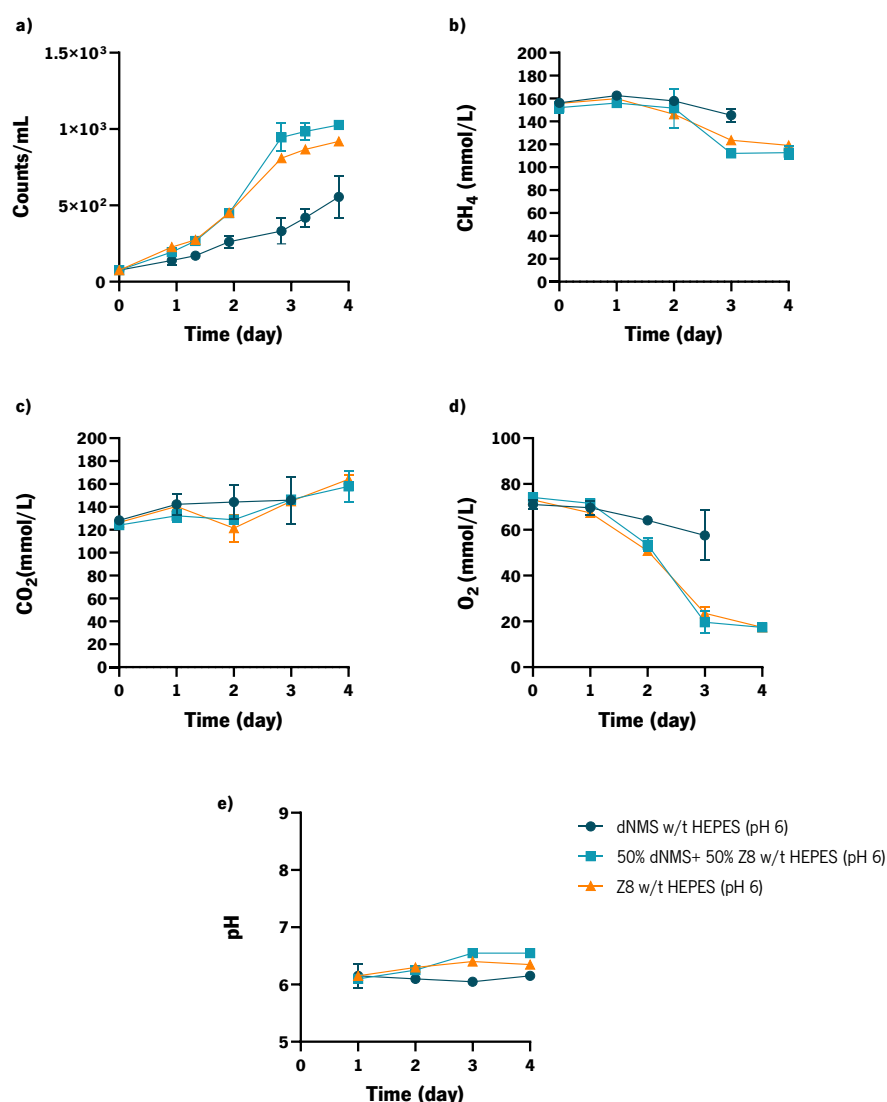


Figure 9. Effect of different growth media (dNMS, Z8, and 50 % (v/v) dNMS+Z8) under optimum pH (pH6) on a) growth, b) CH₄ variation (mmol/L), c) CO₂ variation (mmol/L), d) O₂ variation (mmol/L), and e) pH variation of *Methylococcus* sp. cultures. Error bars indicate minimum and maximum values observed (n=2). ● – dNMS without HEPES (pH 6); ■ – 50 % (v/v) dNMS + 50 % (v/v) Z8 without HEPES (pH 6); ▲ – Z8 without HEPES (pH 6).

Analysing the results of Figure 9, it can be noted that *Methylococcus* sp. sp. is also able to grow in every medium tested. Moreover, Z8 media and 50 % (v/v) dNMS + 50 % (v/v) Z8 provide the best biomass concentration, reaching 919.08 ± 2.6 and 1027.00 ± 8.07 counts/mL on day 4, respectively. Interestingly, dNMS medium had the lowest biomass concentration, only attaining 555.30 ± 97.89

counts/mL during the same growth period. A possible explanation for this result may be related to the composition of the media itself. Even though, dNMS has more macronutrients, such as K, Mg and Fe, it is important to note that Z8 medium contains both ammonium (NH_4) and nitrate (NO_3) as nitrogen sources in its composition, while dNMS only has NO_3 . These compounds are very important to methanotrophic bacteria since they are a source of nitrogen, which is a key component of amino acids, pyrimidines and purines, NAD, and amino sugars. Type I methanotrophs have shown to be capable of using both forms of N (Hu & Lu, 2015). Therefore, the higher growth observed in Z8 medium and 50% (v/v) dNMS + 50% (v/v) Z8 medium could be related to the fact that Z8 medium shows higher concentrations of NH_3 and has a surplus of N with NO_3 when comparing it to dNMS. Moreover, according to Boiesen et al. (1993), in methanotrophic growth, when NH_4 replaces nitrate as the nitrogen source, carbon uptake is more effective. This fact was explained by the author as lower energy consumption is needed to consume ammonium, when compared to nitrate. This could reinforce the fact that the growth observed in Z8 medium and 50% (v/v) dNMS + 50% (v/v) Z8 medium could be due to the quantity of N that is available, since in Table 11, there is higher consumption of CH_4 in these media. Therefore, the higher nitrogen presence in these media could lead to a higher growth of the methanotrophic bacteria. Additionally, it also shows that the concentrations of O_2 suffer a more significant decrease in Z8 medium than in dNMS, while CO_2 concentration experience a higher increase, concordant with CH_4 profile and the metabolism of methanotrophic bacteria. When observing μ_{max} , productivity and x_{max} it is possible to denote that these values are higher in 50% (v/v) dNMS + 50% (v/v) Z8 medium, followed by Z8 medium and dNMS medium, showing to in concordance with the results above mentioned.

Table 11. Effect of different growth media and pH values on the maximum growth rate (μ_{max}), global biomass productivity (Productivity), maximum biomass concentration (x_{max}), rate of CO_2 , rate of O_2 , and rate of CH_4 of *Methylococcus* sp. Negative values in rate represent gas consumption, while positive values represent its production. The values are the mean of two different assays ($n = 2$) with the deviation representing the maximum and minimum values obtained

Condition	μ_{max} (Day^{-1})	Productivity (Counts.mL/daily)	x_{max} (Counts/mL)	Rate CO_2 (mmol.L/daily)	Rate O_2 (mmol.L/daily)	Rate CH_4 (mmol.L/daily)
dNMS without HEPES (pH 6)	0.65 ± 0.26	125.00 ± 25.53	555.3 ± 97.88	4.41 ± 3.26	-3.38 ± 2.25	-2.72 ± 0.44
50% NMS + 50% Z8 without HEPES (pH 6)	0.85 ± 0.01	248 ± 2.04	1027.00 ± 8.07	8.48 ± 2.47	-14.20 ± 0.35	-9.84 ± 1.79
Z8 without HEPES (pH 6)	0.70 ± 0.01	219.9 ± 0.67	919.1 ± 2.62	9.42 ± 0.41	-13.75 ± 0.36	-9.15 ± 0.47

In summary, Z8 and 50 % (v/v) dNMS + 50 % (v/v) Z8 media seem to be the most suitable medium for *Methylococcus* sp.. However, since the highest growth in *Synechocystis* sp. LEGE 07211 was observed in Z8 medium, its use should be favoured.

4.3.2.2. *Methylococcus* sp. using different media with 250mM of HEPES at pH 7

In parallel with the experiments of section 4.3.2.1, another study was performed using the different growth media with HEPES buffer (250 mM) at pH 7, the optimal pH for *Synechocystis* sp. LEGE 07211. Under these conditions, as demonstrated in Figure 10, no growth was observed. Moreover, no variation has occurred in CH₄, CO₂, and O₂ concentration. This suggests that these conditions were not favourable for this particular strain. However, Bussmann et al. (2004) tested different buffers (HEPES, MOPS, phosphate-buffered medium, and TES) when culturing methanotrophic bacteria in dNMS and obtained the highest biomass concentration – 712 ± 178 cells ml⁻¹ – using a 10 mM HEPES-buffered dNMS medium. Additionally, Amaral et al. (1998) showed that using NMS medium with 100 mM of MEPS buffer at pH 7 allowed a consumption of CH₄ of approximately 2 nmol CH₄ consumed (g dry wt)⁻¹g⁻¹.

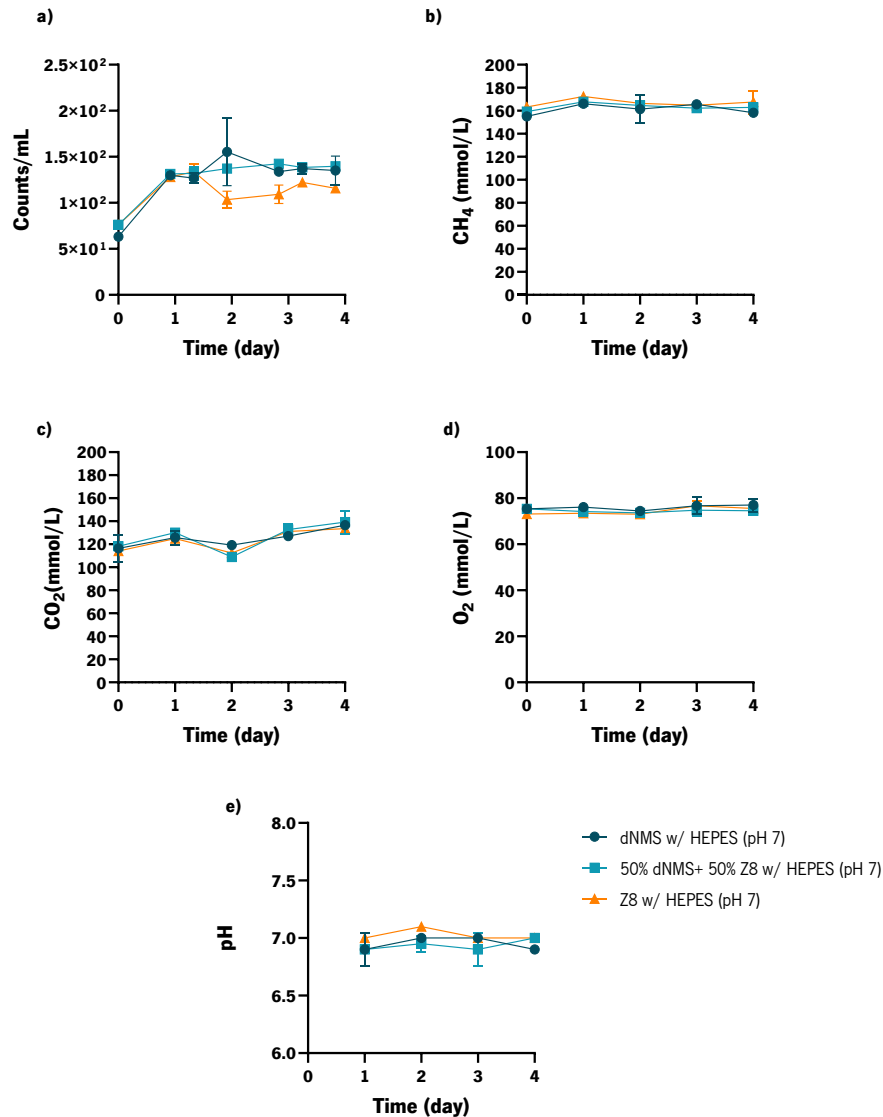


Figure 10. Effect of different growth media (dNMS, Z8, and 50 % (v/v) dNMS+ 50 % (v/v) Z8), under pH 7 and 250 mM of HEPES, on a) growth, b) CH₄ variation (mmol/L), c) CO₂ variation (mmol/L), d) O₂ variation (mmol/L), and e) pH variation of *Methylococcus* sp. cultures. Error bars indicate minimum and maximum values observed (n=2). ● – dNMS with HEPES (pH 7); ■ – 50 % (v/v) dNMS + 50 % (v/v) Z8 with HEPES (pH 7); ▲ – Z8 with HEPES (pH 7).

Therefore, the next step was to test lower concentrations of HEPES – as detailed in section 4.3.2.3 – in order to understand if this strain was being inhibited by the high concentrations of HEPES applied.

4.3.2.3. *Methylococcus* sp. using different concentrations of HEPES buffer

Based on the results of sections 4.3.2.1 and 4.3.2.2, 3 different concentrations of HEPES were selected, keeping in mind that the goal was to use these conditions in co-cultures with *Synechocystis* sp. LEGE 07211. Moreover, it is important to have in consideration that a balanced solution needed to be found, that didn't inhibit *Methylococcus* sp. but, at the same time, that prevented high pH variations due

to any high concentrations of CO₂ that might needed to be use for *Synechocystis* sp. Consequently, the selected concentrations were 30 mM, 60 mM, and 125 mM. Additionally, an assay was also conducted with Z8 medium without any HEPES. All these experiments were done with Z8 medium since it was determined – in section 4.3.2.1 and 4.3.1 – that this medium would be favoured for the co-culture of cyanobacteria and methanotrophic bacteria.

In Figure 11, it is clear that low concentrations of HEPES (30 mM) enable a biomass concentration very similar to Z8 medium without buffer. Contrarily, the use of 60 mM and 125 mM of HEPES doesn't show any relevant growth. Nevertheless, the other conditions behaved as expected in terms of pH.

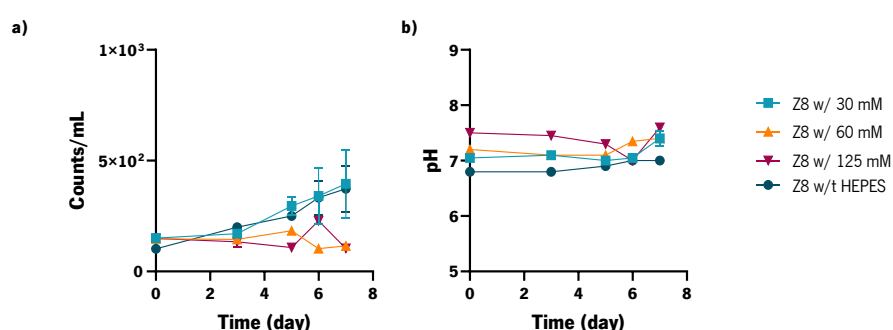


Figure 11. Effects on cultivation of *Methylococcus* sp. using different concentrations of HEPES with Z8 medium and under pH 6 on a) growth and b) pH variation. Error bars indicate maximum and minimum (n=2). ● – dNMS without HEPES (pH 7) ■ – Z8 with 30 mM HEPES (pH 7); ▲ – Z8 with 60 mM HEPES (pH 7); ▼ – Z8 with 125 mM HEPES (pH 7).

Similar results are also presented in Table 12 regarding the growth parameters – μ_{max} , biomass productivity, and x_{max} – obtained for *Methylococcus* sp. cultivation in Z8 with 30 mM of HEPES and without buffer.

Table 12. Effect of different concentrations of HEPES on maximum growth rate (μ_{max}), global biomass productivity (Productivity), and maximum biomass concentration (x_{max}) of *Methylococcus* sp. The values are the mean of two different assays (n = 2) with the deviation representing the maximum and minimum values obtained.

Condition	μ_{max} (Day ⁻¹)	Productivity (Counts.mL/day)	x_{max} (Counts/mL)
Z8 w/ 30mM HEPES	0.18 ± 0.03	35.01 ± 15.37	395.17 ± [286.24;504.10]
Z8 w/ 60mM HEPES	ng	ng	ng
Z8 w/ 125 mM HEPES	ng	ng	ng
Z8 w/t HEPES	0.16 ± 0.04	38.72 ± 10.38	373.12 ± 72.62

ng – no growth.

Based on the range of HEPES concentrations tested in this experiment, it was concluded that *Methylococcus* sp. is inhibited at concentrations above 30 mM. Moreover, the concentration of 30 mM appears to have positive results in terms of growth, which will allow the combination of *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp. in a co-culture using Z8 medium with 30 mM of HEPES at pH 7.

4.4. Experimental Design with co-cultures

The consumption of CO₂ and CH₄ in a co-culture is intimately related to its biomass production and populations evolution. To better understand the tendencies on the consumption of these gases in a co-culture of *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp., an experimental design was performed using a CCD. The effect of 4 independent variables (O₂, CH₄, CO₂, and ratio of *Synechocystis* sp. LEGE 07211) were accessed, whereas the biomass productivity of both *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp., as well as the consumption of CO₂, CH₄, O₂, removal of nitrates, removal of phosphates and total nitrogen were considered and analysed as response variables.

In general, CH₄ and O₂ concentrations decreased (more negative balances) in all conditions, whilst CO₂ balance increased (higher concentrations) (Table 13). This could mean that the consumption of O₂ and production of CO₂ of methanotrophic bacteria happened at a higher pace than the production of O₂ and degradation of CO₂ by the cyanobacteria. However, a study addressing co-cultures of methanotrophic bacteria and microalgae shows O₂ accumulations with a headspace of 60 % CH₄ and 40 % CO₂. It is important to note that the authors did not mention the ratios of the organism that they used (Rasouli et al., 2018).

Overall, CH₄ balances are more negative (higher consumption) in ratios with 25 % *Synechocystis* sp. LEGE 07211, possibly due to the increasing number of methanotrophic bacteria present in these conditions. Additionally, in conditions with 20 % of CO₂, low productivities of *Synechocystis* sp. LEGE 07211 are observed, contrarily to conditions with lower concentrations of CO₂ which tend to have higher productivities of this photosynthetic organism. Furthermore, regardless of their initial ratio, higher productivities of *Methylococcus* sp. are always obtained when compared to *Synechocystis* sp. LEGE 07211. This could be related to the fact that the ratios obtained were in terms of optical density and not number of cells, therefore due to the size of the cells, there is more methanotrophic bacteria present than cyanobacteria in all ratios. This could influence the growth and behaviour of the cultures and promote higher productivities of methanotrophic bacteria. Though, generally, CH₄ doesn't seem to inhibit the growth of *Synechocystis* sp. LEGE 07211 in any concentrations (Table 13).

The removal of nitrates and phosphates appears to be greater when higher productivities of the organisms are obtained. The highest removal efficiency of nitrates achieved 99 % and the lowest 8 %. On the other hand, the removal of phosphates ranged from 23.95 % and 100 %. The content of total nitrogen can be associated to the content of protein (FAO, 2019). In fact, higher nitrogen content tends to be translated in higher protein content. Similarly, to nitrates and phosphates removal, the total nitrogen content are higher when higher productivities of both organisms are achieved.

Table 13. Complete experimental design matrix using CCD for evaluation of the independent variables concentration of O₂, concentration of CH₄, concentration of CO₂, and ratio of *Synechocystis* sp. LEGE 07211 with the corresponding parameter's range. The values obtained for the response variables (productivity of *Synechocystis* sp. LEGE 07211, productivity of *Methylococcus* sp., CO₂ balance, CH₄ balance, O₂ balance, removal of nitrates, removal of phosphates, and total nitrogen) are also presented. Negative values indicate consumption, while positive indicate production.

Code	[O ₂] (%)	CH ₄ (%)	CO ₂ (%)	Ratio of <i>Synechocystis</i> sp. LEGE 07211 (%)	Productivity <i>Synechocystis</i> sp. LEGE 07211 (counts.mL/day)	Productivity <i>Methylococcus</i> sp. (counts.mL/day)	CO ₂ balance (%)	CH ₄ balance (%)	O ₂ balance (%)	Removal of nitrates (%)	Removal of phosphates (%)	Total nitrogen (mg/L)
	Independent variables (X)				Variables of response (Y)							
	x1	x2	x3	x4	y1	y2	y3	y4	y5	y6	y7	y8
1.	-1	-1	-1	-1	4.42	44.99	-15	-97	-9	98	100	513.81
2.	1	-1	-1	-1	0.08	47.51	1	-99	-11	96	100	497.01
3.	-1	1	-1	-1	3.31	45.89	14	-6	-36	99	100	464.21
4.	1	1	-1	-1	1.98	30.23	56	-11	-61	91	100	493.81
5.	-1	-1	1	-1	0.07	25.27	36	-60	-41	37	36.53	25.61
6.	1	-1	1	-1	0.09	25.27	29	-90	-33	32	23.95	38.41
7.	-1	1	1	-1	0.08	19.29	26	-9	-38	35	29.94	62.21
8.	1	1	1	-1	0.08	35.39	36	-15	-53	42	74.97	50.11
9.	-1	-1	-1	1	6.09	6.09	-71	-52	44	98	76.38	144.9
10.	1	-1	-1	1	0.28	10.96	-53	-28	24	78	100	72.7
11.	-1	1	-1	1	2.74	11.89	17	-7	-38	98	100	226.5
12.	1	1	-1	1	5.74	14.33	-20	1	3	94	100	122.7
13.	-1	-1	1	1	0.31	32.59	25	-64	-35	46	48.25	46.5
14.	1	-1	1	1	0.38	7.50	36	-16	-17	8	12.99	0
15.	-1	1	1	1	0.14	35.23	38	6	-41	43	82.54	146.7
16.	1	1	1	1	0.30	38.87	45	-12	-68	76	100	53.2
17.	0	0	0	0	2.71	29.22	66	-30	-55	99	100	176.23
18.	0	0	0	0	1.16	45.85	43	-23	-61	99	100	163.93
19.	0	0	0	0	0.32	24.08	46	-19	-62	79	100	115.33

4.4.1. Effects of response variables

To understand if the models obtained for each response variable were statistically significant, an analysis of variance (ANOVA) is needed. It is important to note that it is not advisable to use a non-statistically significant model. For this evaluation, a F-test is used in order to determine how the response is affected by varying the level of the independent variables. Moreover, the coefficient of multiple determination (R^2) is used to recognize the quality of the fit of the model (Martínez et al., 2011).

In Table 14, the analysis of variance (ANOVA) is exhibited for all response variables (productivity of *Synechocystis* sp. LEGE 07211, productivity of *Methylococcus* sp., balance of CO₂, balance of CH₄, balance of O₂, consumption of nitrates, consumption of phosphates, and total nitrogen). The F-test was used to determine the model's statistical significances and the results showed that all models, apart from balance of O₂ and consumption of phosphates, were statistically significant. However, the fit of the model, which was determined by the coefficient of multiple determination (R^2) was not very appropriate for many of the variables. These lower values of R^2 could be related to the fact that the ratios determined for this experiment were obtained through optical density (750nm). As the size of *Methylococcus* sp. cells are smaller than those of *Synechocystis* sp. LEGE 07211, the ratios were not exactly the same in terms of number of cells. In future works, to avoid these disparities, the ratio of different species should be estimated based on number of cells. Another reason for these discrepancies could be the apparent lack of equilibrium between the consumption of O₂ by methanotrophs and its production by cyanobacteria, which might make the balances of the gas hard to predict in a model. Moreover, in the case of consumption of phosphates, the lower R^2 could be explained by the lower range obtained for this response variable, as observed in Table 13.

Table 14. Analysis of variance (ANOVA) with the percentage of coefficient of multiple determination (R^2), $F_{\text{regression/residuals}}$ (F calculated); $F_{\text{Lack of fit/pure error}}$, and F_{Tab} at a significance level of 10 %, for the responses productivity of *Synechocystis* sp. LEGE 07211 (y_1), productivity of *Methylococcus* sp. (y_2), balance of CO_2 (y_3), balance of CH_4 (y_4), and balance of O_2 (y_5), consumption of nitrates (y_6), consumption of phosphates (y_7), and total nitrogen (y_8).

Variable	R^2	$F_{\text{regression/residuals}}$	F_{Tab}	$F_{\text{Lack of fit/pure error}}$	F_{Tab}
Productivity <i>Synechocystis</i> sp. LEGE 07211 (y_1) = $1.59 - 1.45 x_3 + 0.74 x_1 x_3$	58.22 %	11.2	2.67	1.3	9.30
Productivity <i>Methylococcus</i> sp. (y_2) = $27.92 - 7.27 + 8.40 x_3 x_4$	59.75 %	11.9	2.67	0.6	9.30
Balance of CO_2 (y_3) = $18.68 + 14 x_2 + 21.38 x_3 - 10.37 x_4 - 11.62 x_2 x_3 + 12.50 x_3 x_4$	72.28 %	6.8	2.35	3.6	9.40
Balance of CH_4 (y_4) = $- 33.21 + 28.31 x_2 + 13.44 x_4 + 6.56 x_1 x_4 - 9.81 x_2 x_4$	88.49 %	26.9	2.39	6.1	9.41
Balance of O_2 (y_5) = $89 - 7.44 x_3$	25.74 %	5.9	3.03	56.6	2.62
Consumption of nitrates (y_6) = $70.95 - 27.06 x_3$	71.35 %	42.35	3.03	2.2	9.24
Consumption of phosphates (y_7) = $78.21 + 11.88 x_2 - 22.87 x_3 + 8.88 x_2 x_3$	68.38 %	10.8	2.49	-	9.35
Total nitrogen (y_8) = $179.68 - 18.91 x_1 + 17.53 x_2 - 132.06 x_3 - 83.25 x_4 - 20.59 x_1 x_4 + 18.09 x_2 x_4 + 92.01 x_3 x_4$	98.59 %	109.7	2.34	0.6	9.38

Nevertheless, the balance of CH_4 presented a satisfactory R^2 (88,49 %), which means that 88,49 % of the response variability can be explained by the effect of the model's variables. Besides, $F_{\text{regression/residuals}} > F_{\text{Tab}}$ and $F_{\text{Lack of fit/pure error}} < F_{\text{Tab}}$, meaning that this one-order model is adequate and allows obtaining three-dimensional surface plots (Figure 12.). Likewise, although at a lower level, the balance of CO_2 also presented a satisfactory value of 72.28 % (Figure 13). In the case of the consumption of nitrates and total nitrogen, both presented good R^2 and the f-test was successful as well.

4.4.1.1. CH_4 balance

Based on the results of the CCD experiments (Table 13.), it was possible to identify tendencies for the consumption of CH_4 in the co-culture of *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp. Considering the range of concentrations tested in these experiments, in Figure 12(a) it can be observed that the balance of CH_4 is more negative (higher consumption of CH_4) when lower concentrations of CH_4 (5%) are present at the beginning of the growth, independently of the concentration of O_2 tested. This

means that there is no inhibition to the concentrations tested of O_2 . No literature was also found showing inhibition for these O_2 concentrations. In fact, both organisms have a high affinities for O_2 in these range (Kazbar et al., 2019; Ren et al., 1997). Moreover, *Methylococcus* sp. is described as a type I methanotroph, which prefers low CH_4 environments; therefore, the tendency to a low- CH_4 concentration environment presented in Figure 12. was somehow expected (Graham et al., 1993).

Furthermore, the balance of CH_4 tends to more negative when there is a lower ratio of *Synechocystis* sp. LEGE 07211 (x_4) and higher concentrations of O_2 (x_1) (Figure 12 b). This is expected since O_2 is described in literature as a substrate for the methanotrophic bacteria (Hanson & Hanson, 1996) (Figure 12.). According to CH_4 general equation of methanotrophic bacteria (equation 2), for the consumption of 1 mole of CH_4 , it is necessary 2 moles of O_2 , which means that O_2 could be a limiting substrate under certain conditions. When analysing the conditions in Table 13, it can be noted that when lower concentrations of CH_4 (5%) are present, O_2 is not a limiting substrate (15 % O_2); however, there are also conditions where this compound is a limiting substrate (5 % O_2). In these conditions, the cyanobacteria could produce the rest of the O_2 needed for the consumption of CH_4 by the methanotrophic bacteria.

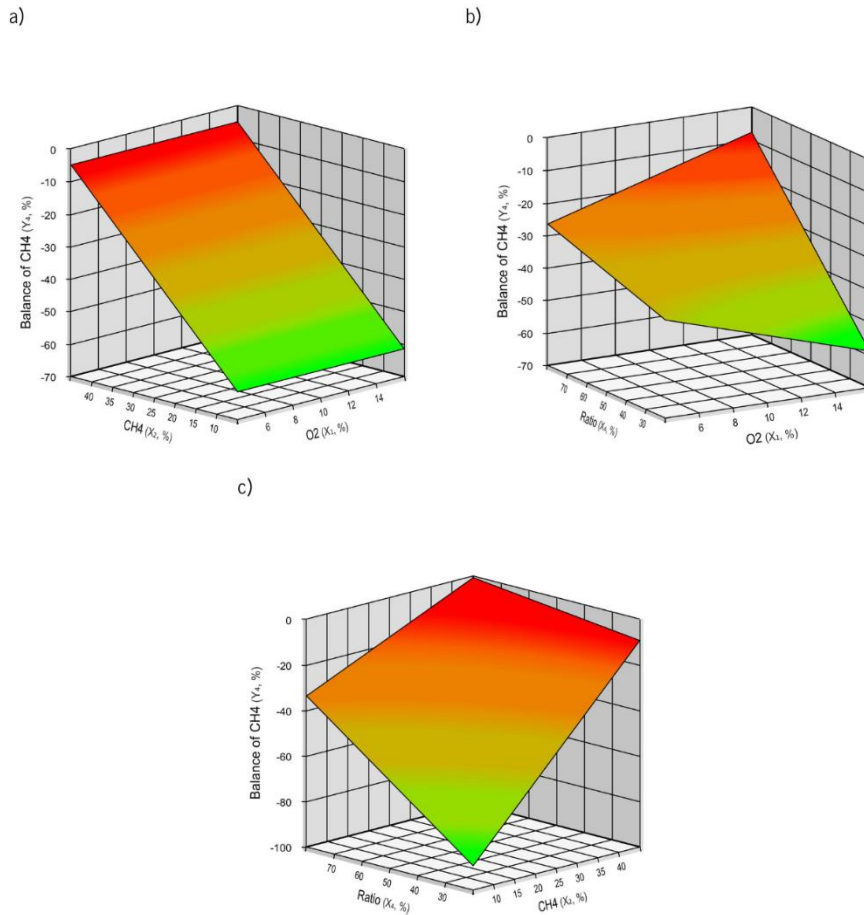


Figure 12. Three-dimensional surface plots for CH₄ balance showing the interactive effects of the tested independent variables on the co-culture of *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp. (a) initial concentration of CH₄ vs. initial concentration of O₂ (b) Ratio of *Synechocystis* sp. LEGE 07211 vs initial concentration of O₂; and (c) Ratio of *Synechocystis* sp. LEGE 07211 vs initial concentration of CH₄.

Lastly, the final three-dimensional surface plot (Figure 12 c) shows that the balance of CH₄ tends to be negatively impacted with lower ratios of *Synechocystis* sp. LEGE 07211 (x₄) in the co-culture and lower initial concentrations of CH₄ (x₂). Higher ratio of *Methylococcus* sp. results in more cells that tend to consume CH₄. Additionally, as mentioned above, *Methylococcus* sp. benefits from low-CH₄ environments, which explains the lower concentrations tendencies (Graham et al., 1993). Ruiz-Ruiz et al. (2020) showed that a ratio of 3:1 (methanotrophic bacteria's consortium: *Scenedesmus obtusiusculus*) with a headspace of 4 % of CH₄ led to the highest degradation of CH₄. However, it was also tested 8 % of CH₄ in the same conditions and lower consumption of CH₄ was observed. This reinforces the fact that these methanotrophic bacteria have higher preferences for lower CH₄ concentrations.

Since only tendencies of the CH₄ balance were found, the next step should be to further decrease the concentrations of CH₄ in order to find the optimum zone where a higher CH₄ consumption (more negative balance) is achieved by the co-culture. Only then, the validation of the model can be performed

and verified. However, it is important to note that concentrations lower than 5 % CH₄, don't have an impactful reduction in emissions, therefore it would be interesting to apply a fed-batch system where the highest amounts of CH₄ would be applied but in smaller quantities at a time. According to the tendencies, the consumption of CH₄ is only associated to the methanotrophic bacteria. However, it is clear that this photosynthetic organism can tolerate the concentrations of CH₄ tested. In future assays, it would be interesting to evaluate the methanotrophic bacteria growth profile and consumption of CH₄ in pure culture under these conditions and understand if the same profiles are visible. If this happens the effect of *Synechocystis* sp. could be neglected. Additionally, as higher number of methanotrophic bacteria were present in all conditions, a repetition of this experimental design but with higher number of cyanobacteria than methanotrophic (determined by number of cells) should be performed in order to understand its effect on CH₄ and if the symbiotic relationship between the organisms is similar to the demonstrated.

4.4.1.2. CO₂ balance

Considering the range of concentrations tested in the experiments of Table 13, a model was generated for CO₂.

In Figure 14 a), it can be observed that the balance of CO₂ tends to be more negatively impacted (higher consumption) when lower initial concentrations of CO₂ (5%) and higher ratios of *Synechocystis* sp. LEGE 07211 (75%) are present. This was expected since higher ratios of *Synechocystis* sp. tend to have a higher number of photosynthetic organisms that will consume the CO₂. Aligned with this result, Martínez et al. (2011) showed that *Synechocystis* sp. prefers lower concentrations of CO₂. Which could explain the reason for higher effect on the consumption of CO₂ under lower concentrations of CO₂ and higher ratios of *Synechocystis* sp.

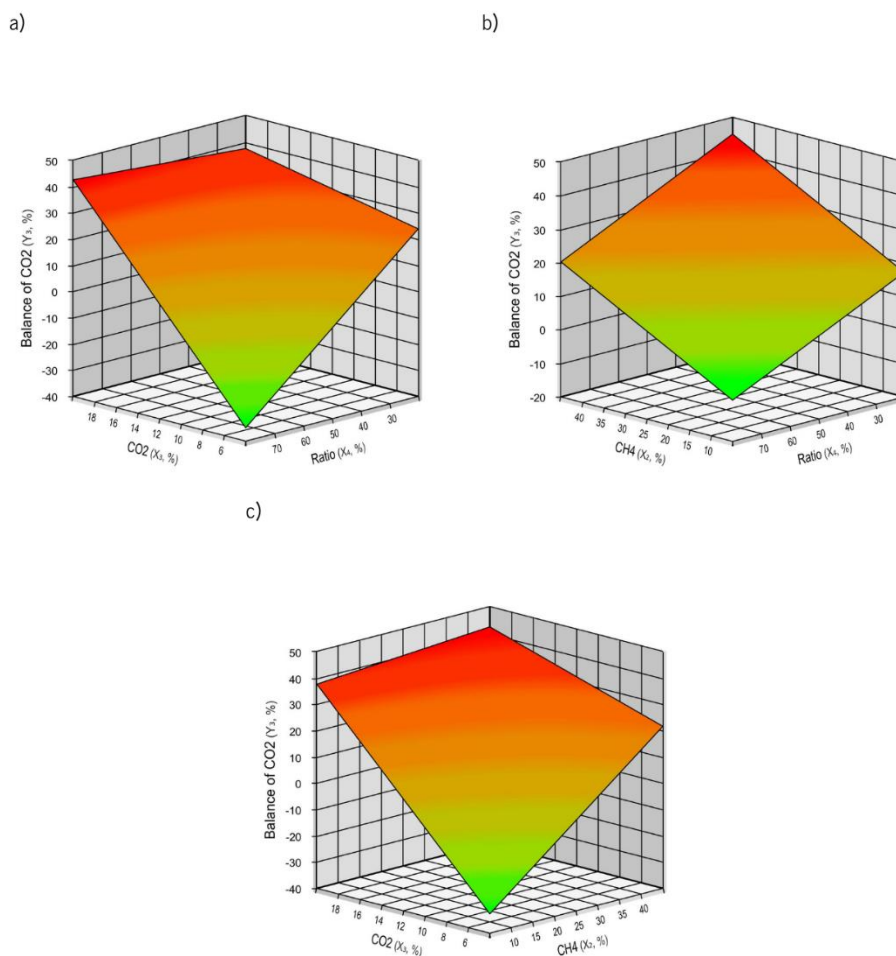


Figure 13. Three-dimensional surface plots for CO₂ balance showing the interactive effects of the tested independent variables on the co-culture of *Synechocystis* sp. LEGE 07211 and *Methylococcus* sp. (a) initial concentration of CO₂ vs. ratio of *Synechocystis* sp. LEGE 07211 (b) initial concentration of CH₄ vs. Ratio of *Synechocystis* sp. LEGE 07211; (c) initial concentration of CO₂ vs initial concentration of CH₄.

Lower concentrations of CH₄ (x_2) and higher ratios of *Synechocystis* sp. LEGE 07211 (x_4) tend to greatly affect the consumption of CO₂. Once again, the consumption of CO₂ is related with the cyanobacteria. Therefore, the balance of CO₂ is expected to be impacted by the number of photosynthetic organisms. Moreover, lower concentrations of CH₄ have shown to be more beneficial for the methanotrophic bacteria, allowing a slower production of CO₂ in order to feed the cyanobacteria (Figure 13 b)).

Lastly, a similar trend is observed in Figure 13 c), which shows that the consumption of CO₂ is affected when lower initial concentrations of CO₂ and CH₄ are present.

As concluded in section 4.4.1.1., the model for the balance of CO₂ only shows tendencies. Therefore, another experimental design should be performed in order to find the optimum region for the consumption of CO₂. Afterwards, the validation of the model can be carried out.

4.4.1.3. Removal of nitrates and total nitrogen

As observed in the model equations of Table 14., there is a negative effect of the initial concentration of CO_2 (x_3) on consumption of nitrates (y_6). This means that lower concentrations of CO_2 enhance the consumption of nitrates. These compounds are sources of an indispensable nutrient (nitrogen), which is used in the synthesis of proteins and amino acids. The degradation of such compounds is associated with the growth of both organisms (Nyerges et al., 2010; Rückert & Giani, 2004). Therefore, if there is higher consumption of nitrates under low- CO_2 environments, this could indicate that there are greater biomass concentrations in the co-culture with less CO_2 (Table 13.). It is important to note that similar relations have been mentioned in section 4.4.1.2.

Higher content of nitrogen is strongly related to higher content of protein (FAO, 2019). Based on the equation of Table 14., the initial concentration of CH_4 (x_4) induces a positive effect on total nitrogen (y_8), while the initial concentrations of O_2 (x_1) and CO_2 (x_3), as well as the ratio of *Synechocystis* sp. LEGE 07211 (x_5) alone, have a negative impact on total nitrogen content. This means that high total nitrogen (more protein) is expected when there are lower concentrations of CO_2 and O_2 and lower ratios of *Synechocystis* sp. LEGE 07211. With this in mind, it appears that total nitrogen (more protein) is strongly enhanced by the methanotrophic bacteria. Moreover, in the range of concentrations tested, there is an increasing tendency of total nitrogen (more protein) when higher concentrations of CH_4 are present. When analysing Table 13. it can be observed that generally higher concentrations of CH_4 are associated to higher productivity. Valverde-Pérez et al. (2020) mentions that the protein content is related to the biomass productivity, therefore it is expected that there is tendency to have more protein in these conditions.

Moreover, there is also a negative effect on total nitrogen resulting from the synergy between the initial concentration O_2 and the ratio of *Synechocystis* sp. LEGE 07211. Contrarily, the model indicates a tendency for a positive impact on total nitrogen content when the initial concentration of CH_4 and the ratio of cyanobacteria interact. Finally, the effect of combining the initial concentration of CO_2 with the ratio of cyanobacteria tends to be positive for total nitrogen.

5. Conclusion and future works

The present work studied the effect of different methods of cultivation of cyanobacteria on the consumption of CH₄, such as with the use of different concentrations of CO₂ + CH₄ and CH₄ alone and co-cultivation with methanotrophic bacteria.

When cultivated with different concentrations of CO₂ + CH₄, the cyanobacteria showed a slight biomass growth, as well as a residual production of CH₄ and consumption of O₂. Moreover, when cultivated only with CH₄, no growth was observed. Nevertheless, a residual consumption and production of O₂ and CH₄, respectively, was also observed in these conditions. Therefore, such experiments suggest no interference of *Synechocystis* sp. in the consumption of CH₄ under the concentrations and conditions tested. However, the residual production observed should be further investigated. The growth observed in all conditions seemed to be related to the consumption of CO₂. Nevertheless, in the future, a triplicate assay of these conditions should be performed in order to understand whether this production of CH₄ and decrease in O₂ concentration is significant or not. Moreover, since the assay with different concentrations of CH₄ was only performed for 12 days, a longer assay should be done to understand if the same profile is verified.

Regarding the preliminary assays performed previous to the co-cultures, Z8 medium at pH 7 was associated with the highest $\mu_{\max}$ for *Synechocystis* sp. ($\mu_{\max}=0.16 \pm 0.01 \text{ day}^{-1}$). Following this, it was attempted to understand if *Methylococcus* sp. could grow in those conditions, and it was concluded that this strain is inhibited by HEPES buffer at 250 mM but is able to grow at 30 mM. Therefore, Z8 medium at pH 7 with 30 mM of HEPES were the conditions determined for the co-culture assays.

In order to test multiple variables at the same time, an experimental design was performed using DCC. In general, *Synechocystis* sp. LEGE 07211 seems to have a negative effect on CH₄ consumption and a positive effect on CO₂ consumption. Moreover, the concentration of CO₂ has a negative effect on the consumption of nitrates. Likewise, CO₂ concentration has also a negative impact on total nitrogen, while CH₄ has a positive effect. However, no optimum region was found for none of the response variables assessed. Therefore, a new experimental design with lower ranges of CH₄ and CO₂ should be tested. After the optimum zone being established, the model can then be validated, where the optimum condition is be carried out in triplicate. Moreover, it would also be interesting to apply a fed-batch system where the highest amounts of CH₄ tested would be applied in the consortium but in smaller quantities at a time, in order to improve CH₄ consumption. Additionally, in future assays, it would be interesting to evaluate the methanotrophic bacteria growth profile and consumption of CH₄ in pure culture under these conditions

and understand if the same profiles are visible. If this happens the effect of *Synechocystis* sp. could be neglected. Also, due to the higher number of methanotrophic bacteria present in all conditions, a repetition of this experimental design but with higher number of cyanobacteria than methanotrophic bacteria (determined by number of cells) should be performed in order to understand its effect on CH₄ consumption and if the symbiotic relationship between the organisms is similar to the observed.

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Annexes

Table A1. Specific compositions of Z8 medium and dNMS media.

Z8 Medium		dNMS Medium	
Nutrient	g/L	Nutrient	g/L
Mg	0,002465	Mg	0,019722
SO ₄	0,016776	SO ₄	0,078979
Na	0,135481	Na	16,92326
NO ₃	0,386586	NO ₃	0,122651
Ca	0,014383	Ca	0,008165
K	0,015295	K	0,107869
PO ₄	0,016901	PO ₄	0,265429
H	0,000178	H	0,003577
CO ₃	0,011888	CO ₃	-
W	0,000204	W	-
O	0,002792	O	2,39E-05
NH ₄	0,000261	NH ₄	-
Mo	7,11E-05	Mo	1,19E-06
Br	0,000806	Br	5,25E-06
I	0,000635	I	-
Co	0,000141	Co	4,95E-06
Cd	0,000707	Cd	-
Zn	0,000652	Zn	2,27E-06
Cu	0,000498	Cu	0,000636
Ni	0,000294	Ni	4,94E-07
Cr	5,33E-05	Cr	-
V	4,99E-05	V	-
Al	0,000545	Al	-
B	0,000542	B	-
Mn	0,000549	MN	8,33E-07
Fe	1,47E-06	Fe	0,000156
Cl	1,84E-06	Cl	0,014476
EDTA	3,7E-06	EDTA	0,000791

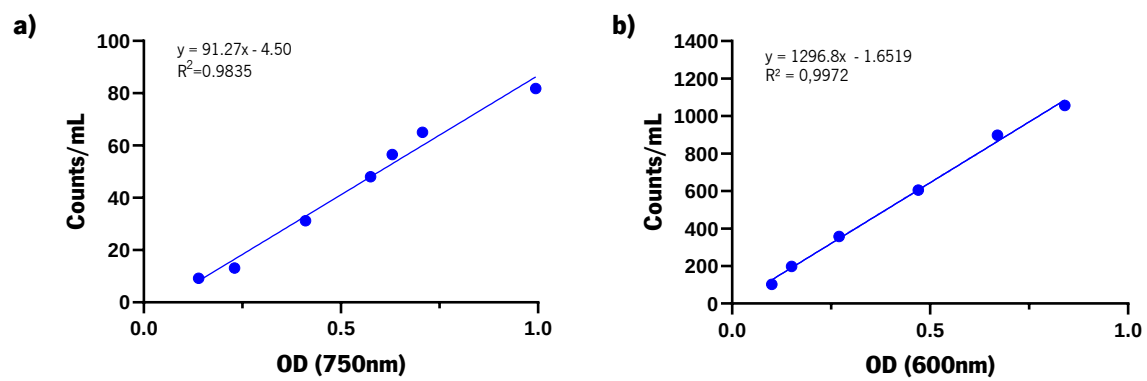


Figure A1. Biomass calibration curve of a) *Synechocystis* sp. and b) *Methylococcus* sp.