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Oxic methane production by cyanobacteria from the Brazilian Pantanal soda lakes

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Thesis presented to obtain the degree of Doctor in Science:
Area: Agricultural Microbiobiology

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Bachelor of Biological Sciences

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

Advisor:
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1. Metanogênese 2. Fosfonato 3. Produção primária 4. Metadados 5. Espectrometria de Massas com Membrana Interna (MIMS) I. Título

Dedicatory

To Beatriz Rosa, Alice Andreote, and Eva-Mai Ionescu
for reminding me that we are born courageous and clever girls
with nothing impossible to our hearts and minds to achieve.
I was lucky to have your adventures inspiring me to not giving up on mine.

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“Never be cruel.
Never be cowardly.
Remember, hate is always foolish and love is always wise.
Always try to be nice, but never fail to be kind.”

The Twelfth Doctor, Doctor Who (Twice Upon a Time, 2017)

SUMMARY

RESUMO

Produção aeróbica de metano por cianobactérias das lagoas salino-alcalinas do Pantanal brasileiro

Metano (CH_4) é um dos principais gases de efeito estufa influenciando significativamente as mudanças climáticas. A principal fonte biogênica de metano em ambientes aquáticos é o processo de metanogênese realizado pelas arquéias, estritamente anaeróbico. Entretanto, recentemente, a produção e emissão pela atividade de bactérias em águas altamente oxigenadas tem sido descrita para diversos sistemas – "Paradoxo de Metano", e cianobactérias são consideradas com um dos principais responsáveis pela produção aeróbica de metano (da sigla em inglês, OMP). O Pantanal é um dos biomas brasileiros, onde a sub-região da Nhecolândia apresenta lagoas salino-alcalinas com níveis elevados de salinidade, alcalinidade ($NaHCO_3$) e pH (>9). Estudos anteriores nessas lagoas correlacionaram elevadas concentrações de metano com aqueles que possuem florações intensas de cianobactéria. Neste trabalho, o objetivo foi explorar a ocorrência de emissões aeróbicas de metano nas lagoas salino-alcalinas do Pantanal usando dados genômicos e metagenômicos, além de ensaios com isolados de cianobactéria para avaliar a potencial atuação direta desses organismos na produção de metano. No primeiro capítulo, com uma análise geral de vias de OMP, o uso de marcadores metagenômicos se mostrou eficientemente satisfatória para iniciar a investigação desses processos. Além de reforçar que lagoas com floração de cianobactéria apresentam elevadas concentrações de metano dissolvido na água. Algumas vias foram exploradas, porém o potencial destas é aparentemente dependente das propriedades químicas das lagoas. Com a via da liase C-P se mostrou interessante nas lagoas com florações, análises mais robustas focaram nesse processo, sendo demonstrado no capítulo dois. Aqui foi identificada uma comunidade microbiana capaz de realizar essa via, com fatores favorecendo sua ocorrência, como as concentrações de nitrogênio e ferro. Entretanto, cianobactérias não são os principais responsáveis, tendo provavelmente um papel secundário nesta OMP. Por fim, no capítulo três foi realizado um monitoramento dos isolado cianobacterianos das lagoas salino-alcalinas. Foi encontrada uma produção de metano exclusiva pelas cianobactérias, altamente relacionada com a fixação de carbono. Assim, compreende-se nesse trabalho que é incerto afirmar que a OMP seja uma grande contribuinte para as emissões de metano nas lagoas salino-alcalinas do Pantanal. Porém, as cianobactérias são capazes de produzir metano, incluindo àquelas que formam floração naturais, e podem indiretamente influenciar em outras fontes de emissão. Dessa forma, essa relação deveria ser investigada pelo seu impacto real nas emissões de gases de efeito estufa.

Palavras-chave: Metanogênese, Fosfonato, Produção primária, Metadados, Espectrometria de Massas com Membrana Interna (MIMS)

ABSTRACT

Oxic methane production by cyanobacteria from the Brazilian Pantanal soda lakes

Methane (CH_4) is one of the main greenhouse gases significantly impacting climate changes. The main biogenic source of methane in aquatic environments is archaeal methanogenesis, strictly anaerobic. However, recently, bacterial productions and emissions from highly oxygenated waters have been described in many systems – "Methane Paradox", and cyanobacteria are considered as one of the main responsible organisms for the called oxic methane production (OMP). The Pantanal is one of the main Brazilian biomes, and the Nhecolândia sub-region encloses saline/alkaline or soda lakes with high levels of salinity, alkalinity ($NaHCO_3$), and pH (>9). Previous studies on the Brazilian soda lakes have correlated elevated methane concentrations with the ones presenting intensive cyanobacterial blooms. In this study, we aim to explore occurrence of oxic methane production in the Pantanal soda lakes using metadata approaches from genomics and metagenomics and isolates bioassays to evaluate cyanobacteria' potential for direct participation in methane production. Our first study on general OMP pathways applying metagenomics makers was satisfactory efficient to begin our analysis, and reinforced that the lakes with cyanobacterial blooms have higher methane concentrations in their water. Although several pathways were searched, their potential to occur depends on the lake's chemical properties. As the 'C-P lyase' pathway had an interesting potential on lakes with blooms, we performed a deeper analysis, which is reported in chapter two. Here, we in fact verified that there is a microbial community able to use this pathway on the lakes, with factors, such as nitrogen and iron concentration favoring it. However, cyanobacteria are not the main responsible for it, so their role would be probably secondary in this case. Lastly, the study described in chapter three focused on monitoring cyanobacterial strains isolates from the soda lakes. We found methane production exclusive to cyanobacteria and highly correlated to carbon fixation processes. Therefore, we understand in this work that OMP on the soda lakes is unlike to be the major methane production emissions contribution. However, the cyanobacteria are able to produce methane, including the naturally occurring blooms, and also can indirectly influence other sources, which should be investigated for their real effect on greenhouse gas emissions.

Keywords: Methanogenesis, Phosphonate, Primary production, Metadata, Membrane Inlet Mass Spectrometry (MIMS)

1. GENERAL INTRODUCTION AND JUSTIFICATIVE

Methane (CH_4) is the second most important greenhouse gas impacting climate change. Having a considerable contribution to 10-40% of the global warming process, it shows a ca. 25 times higher warming potential than CO_2 (Bridgham et al., 2013; Tang et al., 2014). The main studied biogenic pathway for methane production involves microorganisms from the Archaea domain called methanogenic archaea (Thauer et al., 2008). However, recent studies have demonstrated that terrestrial plants, algae, fungi, and bacteria hold a variety of mechanisms to release methane as a subproduct of their metabolism, and thus constitute alternative sources of methane to the atmosphere (Keppler et al., 2009; Lenhart et al., 2012, 2016; Sosa et al., 2017).

Cyanobacterial contribution to methane emissions has been recently suggested in some environments where classical pathways of methanogenesis would hardly happen due to high concentrations of oxygen in the surroundings. According to the *'methane paradox'*, there can be an oversaturation of CH_4 in oxic surface and deeper water of oceans and lakes where oxygen concentrations are high (Grossart et al., 2011; Tang et al., 2014, 2016). In both marine and freshwater environments, the role of oxic methane production (OMP) has been attributed to cyanobacteria, especially to bloom-forming ones in surface waters (Bižić et al., 2020; Hartmann et al., 2020; Teikari et al., 2018).

Lately, some interesting works reveal OMP pathways that are related to cyanobacteria. Among them, we can cite the demethylation of organic compounds, such as the "C-P lyase" pathway for methylphosphonate (MPn) (Karl et al., 2008), and the usage of dimethylsulfoniopropionate (DMSP) (Damm et al., 2010). Even more recent studies have focused on the activity of nitrogenase-like enzymes that have been shown to possibly lead to methane production by an unknown process (North et al., 2020). Another potential pathway has been described for freshwater systems, which is related to photosynthetic activity, whereby carbon dioxide fixed by cyanobacteria can be converted to CH_4 (Bižić et al, 2020). Thus, cyanobacteria would be an important biological source of methane, contributing to greenhouse gas emissions to the atmosphere and thus climate change.

The Brazilian Pantanal sub-region known as Nhecolândia presents a total area of 24,000 km^2. In there, we can find about 600 shallow soda lakes are found with high levels of salinity, $NaHCO_3$, and pH (> 9), being considered as extreme environments (Guerreiro et al., 2019). Bloom-forming planktonic cyanobacteria tend to dominate these saline/alkaline environments, and render them extremely productive due to their high photosynthetic rates (Almeida et al., 2011; Malone et al., 2012a; Santos et al., 2018; Sorokin et al., 2014a). Barbieiro *et al.* (2018) described elevated concentrations of CH_4 in surface waters of the Pantanal soda lake, especially from the ones with blooms of cyanobacteria. In their study, they showed an average of 0.8 mmol m^{-2} per day in lakes with no cyanobacterial bloom, meanwhile a range from 56-74 mmol m^{-2} d^{-1} was found on the presence of cyanobacteria, configuring 94% of the yearly methane emissions from the Pantanal lakes.

Even though there is a presence of anaerobic methanogenesis in the sediment, in theory, oxygen production from the cyanobacteria blooms would not allow for elevated concentrations of methane due to high oxidation rates (Tang et al. 2014). Approximatelly 90% of methane emissions at the Pantanal soda lakes occur during periods of oxygen supersaturation in the water (Barbiero *et al.* 2018). Therefore, we hypothesize that other alternative pathways for methane production are likely happening in these environments, contributing for the elevated concentrations previously described.

In this context, we suggest that cyanobacteria blooming in soda lakes contribute to methane production through OMP pathways. Investigating these metabolisms will help to better understand methane release from oxygen saturated waters. Further, it allows to better evaluate and quantify the carbon flux to the atmosphere, and moreover to explore new microbial groups related to methane production. This reinforces the importance of evaluating the most diverse sources of greenhouse gases, which relies on a large-scale environmental condition, and also might indicate possible alternatives to avoid massive accumulation of methane and to mitigate its emission to the atmosphere.

1.1. Objectives

1.1.1. General

This study aims to explore oxic methane production in soda lakes of the Brazilian Pantanal, and characterize the cyanobacteria ability to perform these processes. It also aims on contributing to improve our understanding of the referred oxic methane formation pathway in relation to the metabolism of different primary producers based on genomics and metagenomics approaches, together with *in vitro* bioassays.

1.1.2. Specifics

I. Identify and analyze the potential for oxic methane production pathways on soda lakes, with emphasis on cyanobacterial contribution from water data on environmental conditions;
II. Explore microbial community structure and composition for the ‘C-P lyase’ pathway using genomic and metagenomic data;
III. Understand likely drivers of the ‘C-P lyase pathway’ potential to be adding to the methane budget of soda lakes;
IV. Measure real-time methane production rates of cyanobacteria in culture conditions from soda lakes.

1.2. Literature Review

1.2.1. Brazilian Pantanal soda lakes

Pantanal is one of the six major Brazilian biomes, mainly located on the central west area of the country, trans-passing the borders to Bolivia and Paraguay (approximately 210,000 km^2). (Guerreiro et al., 2019). Characterized as the largest wetland area in the world, the Pantanal is sub-divided in 11 regions only in the Brazilian territory, occupying around 140,000 km^2 of the States of Mato Grosso and Mato Grosso do Sul (Santos, 2013). The subregion Nhecolândia corresponds to 19.5% of the total area of the Brazilian Pantanal and it projects a dense hydrological area with approximately 10,000 shallow lakes (Genuário et al., 2018a). Regional climate is Awa, which is tropical with dry winters (from April to October) and rainy summers (from November to March), annual mean temperature of 25.5º C (with daylight period up to 40º C), and annual mean precipitation between 1000-1500 mm (Hamilton, 2002).

The wetlands ecological functioning is maintained by the seasonal flooding cycle from the Upper Paraguay River, shifting between inundation and desiccation (Guerreiro et al., 2019; Hamilton, 2002). Mostly at the Nhecolâdia's subregion, the lakes are shallow (up to 2 m), hydrologically closed, presenting saline/alkaline characteristics, also denominated in literature as "soda lakes". They are completely isolated from the river and the flooding cycle due to its surroundings of sandy ridges covered by trees (Furian et al., 2013; Guerreiro et al., 2019; Santos, 2013).

These lakes have several classifications that can diverge according to limnology and functional biochemistry, including pH and electric conductivity (EC) (Barbiero et al., 2008; Bergier & Assine, 2016; Genuário et al., 2018a; Malone et al., 2012b; Santos, 2013; Santos & Sant'Anna, 2010). Soda lakes present high alkaline pH (9-11) and EC (>2,000 µS cm^{-1}), showing a predominance of autotrophic metabolism with high primary activity dominated by cyanobacteria and restricted biodiversity (Santos, 2013). On the other hand, freshwater lakes show an acidic pH tendency (5-7.4), very low EC (<1,000 µS cm^{-1}), much more diverse in terms of flora and fauna, having an autotrophic metabolism, however, above the water line with predominance of macrophytes (Bergier & Assine, 2016). There are also intermediate lakes that are dominated by a heterotrophic metabolism and connected to the freshwater lakes on the flooding seasons, normally showing a low pH (5-7). However, the pH increases during the dry periods (up to 9) transforming its characteristics to somewhat closer to the soda lakes, including the dominance of cyanobacteria species (Santos, 2013).

The formation of soda lakes happens after prolonged evaporation periods during summer, when drought can be occurring (Malone et al., 2012b). The ecological typologies of the Nhecolândia lakes are affected by summer rainfalls, leading to temporal changes. In figure 1, a model is presented for possible lake function interchanges with the regional, based on the EC and pH values relating alkalinity and nutritional, hydrology as described by Bergier & Assine (2016).

According to the model (Figure 1), there is a cycle from freshwater lakes turning into a soda lake, when alkalinity and nutrients availability increases, or an intermediary status if only alkalinity increases. Following this idea, soda lakes can change into an intermediary type if only the last one decays. All these changes are linked to how long are the periods of drought and flood; long-term drying could also lead completely disappearance of the lake.

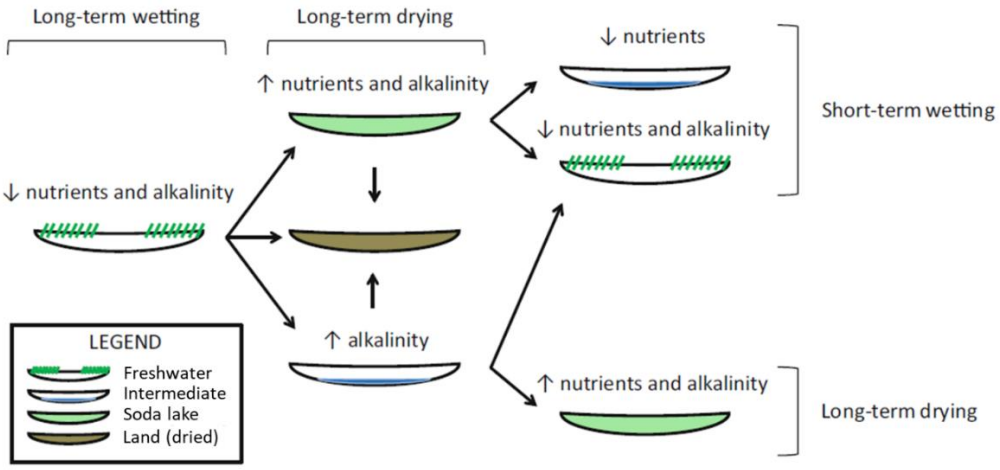

Figure 1. Hydrological model for ecological states of the Nhecolândia soda lakes. Retrieved and adapted from Bergier & Assine (2016).

Concerning salt concentrations, there is an independency of salinity levels for each type of lake. This is explained by the control of the water flow around them separately, allowing for such different hydrological conditions (Furian et al., 2013). Strong evidence indicates that the hydrochemistry, biological diversity and productivity of the soda lakes are controlled by the cemented soil beneath the lake from the groundwater flow and local rainfalls (Hamilton, 2002; Malone et al., 2012b). While soda lakes have much less interference from the freshwater system because of their isolation created by the sand ridges and low permeability in the soil, preventing ion concentrations to dilute; freshwater lakes have a regular water cycle with a less pronounced evaporation (Barbiero et al., 2008). Therefore, salinity is naturally maintained in accordance with the drainage situation.

The alkaline/saline conditions can make the lakes very restrained in their biological diversity, including microorganisms. Sometimes called extremophiles, these organisms can overcome the uncommon chemistry having a special enzymatic machinery that allows them to survive and function at high pH levels (Bergier et al., 2012). For example, photosynthetic activity is very efficient in the soda lakes, therefore, cyanobacteria and diatoms are commonly predominant organisms (Malone et al., 2012b). Moreover, the hydrological characteristics of the Pantanal wetlands might also affect microbial community composition of the lakes, where diversity is commonly lower during drought periods as the extreme conditions increase (Malone et al., 2012b).

1.2.2. Cyanobacteria under saline/alkaline conditions

Cyanobacteria phylum from the domain Bacteria represents primitive organisms on the planet, having fossil registers dated from approximately 3.5 billion years ago (Paerl & Paul, 2011). These microbes can perform oxygenic photosynthesis, which means that they use water (H_2O) molecules to produce oxygen (O_2) (Flores et al., 2015). Cyanobacteria are able to combine different metabolic strategies on several habitats, allowing them to inhabit a broad range of environments (Oren, 2014). Metabolically, these bacteria participate in diverse biogeochemical cycles for several elements, mainly carbon, nitrogen, and phosphorous (P) (Paerl & Paul, 2011).

Phosphorous and nitrogen are both related to cyanobacterial bloom formation, stimulating their growth when found in elevated concentrations (Yan et al., 2017). Several factors can cause blooms, such as excessive organic matter, strong pluviosity periods followed by a drought time, climatic events such as 'El Niño' and 'La Niña', anthropogenic activities near water bodies; whereas, they are all related to high nutrient availability and temperature (25-28ºC) (Carey et al., 2011; Huisman et al., 2018).

Cyanobacterial carbon fixation through photosynthesis, together with pericellular carbonic anhydrase activity can lead to water alkalinization (Almeida et al., 2011). When CO_2 is captured, it can decrease the dissolved HCO_3^- in the water, releasing OH^-, and increase the formation of CO_3^{2-}. As the soda lakes have low concentrations of calcium (which, instead would lead to the formation of $CaCO_3$), the pH ended up increasing, contributing to their alkaline conditions (Almeida et al., 2011).

Even though they can be hostile to several forms of life, soda lakes are highly productive habitats in different tropical localities, like Australia, Brazil, Ethiopia, Kenia, and Tanzania (Hamisi et al., 2017). Relishing on the low competitiveness, the dominance of cyanobacteria in these lakes is due to their eco-physiological plasticity and adaptability mechanisms; for instance, osmotic and intracellular regulation, photosynthesis, mobility and floatability in the water column (Hamisi et al., 2017; Schagerl, 2016). For some taxa, there is also the presence of differentiate cells called heterocyst and akinetes, functioning for nitrogen fixation and resistance structure, respectively (Flores et al., 2015).

Cyanobacterial dominance is not restrained to specific groups though, and quite a diversity of them were previously described in these environments – unicellular, filamentous, bloom-forming, benthonic, with and without heterocysts (Genuário et al., 2018; Sorokin et al., 2014). Unicellular representatives include the genera *Synechoccocus, Synechocystis* and *Chroococcus* (Andreote et al., 2014; Santos & Sant'Anna, 2010; Schagerl, 2016). Filamentous diversity comprehends *Arthrospira* sp., *Pseudanabaena* sp., *Oscillatoria* sp. and *Aphanocapsa* sp. (Schagerl, 2016). In Southeastern Siberia, *Gleitlerinema* sp., *Spirulina major*, *Leptolyngbya* sp., and *Phormidium etoshii* were described from the shore of Lake Kiran (Burganskaya et al., 2018). In the Brazilian Pantanal, literature shows the presence of *Cyanobacteriaceae, Spirulinaceae, Nostocaceae* and *Rivulariaceae* families (Santos &

Sant'Anna, 2010). In addition, two new non-heterocyst cyanobacterial genera were described from the Pantanal soda lakes, *Pantanalinema* and *Alkalinema* (Vaz et al., 2015).

However, most of the primary production stems from the photosynthetic activity of planktonic strains which dominates the water column, and develop as blooms (Sorokin et al., 2014a). The main bloom-forming genera at soda lakes are *Anabaenopsis Arthrospira,* and *Cyanospira* (Andreote et al., 2018; Burganskaya et al., 2018; Genuário et al., 2016; Sorokin et al., 2014a). *Arthrospira* sp. are highly tolerable to high alkalinity levels, which can explain their dominance on the soda lakes blooms (Burganskaya et al., 2018). In case of decreasing the salinity levels, the dominant strain shifts to the genera *Anabaenopsis* sp. which can also well adapt to the extreme physical and chemical conditions (Santos et al., 2018). Previous works have described alternance or co-dominance of both groups as responsible for the blooms, considering seasonal factors as nutrient input, and grazing by animals (Santos et al., 2018; Schagerl, 2016).

1.2.3. Methane cycling

The methane cycling in the ecosystem reflects the balance between production and consumption. About 40% of the produced methane reaches the atmosphere and the rest is oxidized (Whiticar, 2020). Wetlands greatly contribute about 20-39% of the global methane emission (102-182 $TgCH_4\ yr^{-1}$), mostly not related to anthropogenic activities (Conrad, 2009; Saunois et al., 2019). In contrast, other inland water systems have a lower contribution – freshwater systems (lakes, ponds, reservoirs, streams, and rivers) may be responsible for 6-16% of the natural emissions (117-212 $TgCH_4\ yr^{-1}$), which is much higher compared to oceans (biogenic, geological and hydrate emissions from open and coastal oceans) of less than 1% (9-22 $TgCH_4\ yr^{-1}$) (Saunois et al., 2019).

The methane input to the atmosphere depends on the environmental conditions and source of gas, which can lead to different emission rates (Saunois et al., 2019). Atmospheric methane achieved 2000 ppb in 2021, which corresponds to 2.6 times more than predicted from the pre-industrial era (Bizic, 2021). One of the main methane sources is from anthropogenic activities in livestock enteric fermentation; biomass, solid waste and landfills burning; fossil fuel production and distribution; rice cultivation; and coal mining (Bridgham *et al.*, 2013; Enzmann 2018). Natural sources of methane come from organic matter decomposition through thermogenic, pyrogenic, and biogenic process (Tang et al., 2016). Those include, wetlands, inland water systems, volcanos, seeps, micro seepage, termites, thawing permafrost, and oceanic sources (Lenhart et al., 2015). The biogenic production of methane is called methanogenesis and it will be described in more details in the next section.

Abiotic methane oxidation results from photochemical hydroxyl radical action, followed by the microbial methanotrophic activity as methane sinking (Conrad, 2009; Whiticar, 2020). Methanotrophy refers to microbial methane consumption by chemoautotrophic bacteria or archaea,

oxidizing CH_4 either under aerobic or anaerobic conditions, respectively. Biological oxidation steps can use methanol, formaldehyde and formate as sequential intermediary substrates (Borrel et al., 2011). Similar to methanogenesis, this process has a main enzyme activity that is used as a genetic marker for this process. Methane monooxygenase (*mmo*X) corresponds to a complex that catalyzes the conversion of CH_4 to methanol in soluble and particulate forms (Borrel et al., 2011). For a few representatives, *pmo*A gene can also be a functional marker for methanotrophic activity detection (Kharitonov et al., 2021).

Methane consumption in aquatic environments can account for up to 30-99% of what is produced, reducing the net of methane emitted to the atmosphere (Whiticar, 2020). In aquatic environments with large depth, methane produced in the sediment is consumed by methane oxidizers when the gas is transported through the water column. Therefore, the region inhabited by methanotrophs acts as a barrier controlling the amount of methane that reaches the atmosphere (Kharitonov et al., 2021).

In pelagic systems, methane is transported through the water column via diffusion, ebullition, and plant-mediated transport through aerenchyma (Bridgham *et al.*, 2013). Methanotrophy is affected by the specific environmental conditions and methane is transported through the water column from the sediment – only diffusion and advection from seasonal mixing stimulate methanotrophs; ebullition and plant-mediated fluxes exclude it (Borrel et al., 2011). This happens because via the last two transport pathways, the gas can bypass the aerobic methanotrophic areas in the water (Bridgham *et al.*, 2013).

1.2.4. Biogenic methane production

Concerning biogenic sources of methane, from restricted to Archaea, nowadays are known to be performed by other microbial groups as well. Ordinarily, methane production is only attributed to archaeal methanogenesis, which is restricted to anaerobic conditions, using end products of organic matter fermentation as substrates (Evans et al., 2019). The presence of oxygen can inhibit some enzymes and cofactors of methanogenesis sensitive to it (Borrel et al., 2011). In addition, in terms of thermodynamics, the fermentative degradation of organic matter mainly happens in the absence of oxygen as electron acceptor, so it can be energetically favorable to use other substrates (Evans et al., 2019).

Archaeal methanogenesis is classified according to the precursor substrates for methane. While acetoclastic methanogenesis uses acetate to form CH_4 and CO_2; hydrogenotrophic methanogenesis uses hydrogen (from formate, ethanol and secondary alcohols) and carbon dioxide to form CH_4 and H_2O; and methylotrophic, when methyl-groups are the source of methane to the atmosphere (Borrel et al., 2011; Enzmann et al., 2018). The main environments for this activity are

livestock animals' rumen, rice fields, wetlands and anaerobic sediments (Conrad, 2009; Thauer et al., 2008). The final enzyme to act on the breakage of these substrates is the methyl-coenzyme M reductase, which can be translated from the gene *mcr*A, and until today, is known to be exclusive to archaeal genomes (Evans et al., 2019).

Contradicting the preconditions for archaeal methane production, literature has reported on methane saturation in oxygen-rich surficial waters (around 7-9 m) – the so called 'Methane Paradox' (Tang et al., 2016). From this event, new possibilities were explored in aquatic environments in order to understand which other organisms can contribute to methane emissions leading to methane accumulation even in the presence of oxygen. Among explanations for this paradox, there are physical transportn from littoral production; non-enzymatic reactions on sulfur compounds; products released during reactive oxygen species formation; methanotrophic photoinhibition on surficial zones; gene expression for O_2 consumption to avoid its effect over methanogenic enzymes; and others (Bridgham et al., 2013; Tang et al., 2016; Repeta & Boiteau, 2017).

Some studies point to microbial interactions in anaerobic microenvironments to support archaeal methanogenesis. Sediment aggregates, gastrointestinal tracts and fecal pellets of zooplankton and fish (Grossart *et al.*, 2011; Tang et al., 2014; Wang *et al.*, 2021), are commonly cited as microenvironments with little or none oxygen present, so archaea could be acting inside of them. However, the anoxic condition does not remain long enough for this process to be mainly contributing for the methane paradox.

Moreover, recent investigations on microbial activities in these supersaturated surface waters have revealed new pathways. Demethylation of organic compounds – methyl-phosphonate (MPn), demethylsulphopropionate (DMSP), methylamines (TMA); nitrogenase-like enzymes activities, conditionate to the presence of iron (Fe); photopigments degradation sub-products; and methane production during carbon fixation by photoautotrophic organisms, are the main 'oxic methane production' (OMP) pathways studied to understand the 'Methane Paradox' (Bižić-Ionescu et al., 2018; Bižić, Klintzsch, Ionescu, Hindiyeh, & Günthel, 2020; Damm et al., 2010; Karl et al., 2008; Kharitonov et al., 2021; North et al., 2020; Perez-Coronel & Beman, 2021; Q. Wang et al., 2021). All these alternatives are likely to happen at zones with high concentrations of oxygen, relatively closer to the interface water-air, and therefore the gas released would travel a short distance from oxygenated regions to achieve the atmosphere, increasing the methane emission budget significantly (Tang et al., 2014).

1.2.5. Methyl-phosphonate demethylation pathway

Phosphorous is a macronutrient essential for microbial growth and metabolism, as a structural component of phospholipids, polysaccharides, nucleic acids, proteins and enzymatic

cofactors (Mcgrath et al., 2013). Due to its easy assimilation, inorganic phosphorous (Pi) is the main form of P to be obtained by microorganisms when available (Karl et al., 2008). However, organic phosphorous (Po) molecules are used as alternatives when Pi is depleted in the environment. Among Po forms, there are phospho-esters (C-O-P bond) and phosphonates (C-P bonds) (Chin et al., 2016).

Phosphonates (Phn) correspond to a significant fraction of Po in aquatic environments, representing 25% of the dissolved molecular organic phosphorous in oceans (Gomez-garcia et al., 2010). In nature, Phn occurs in aquatic systems as metabolites of particulate organic matter and microbial cell membrane (Ren et al., 2015); and synthetically, they can be found as chemical components of pesticides, detergents, antibiotics and flame retardants (Studnik *et al.*, 2015). Characterized by the carbon-phosphorous (C-P) bond, the simplest form of Phn is the methyl-phosphonate (MPn), which can be a precursor for more complex Phn structures (Karl et al., 2008).

There are three enzymatic pathways to degrade the C-P bond, hydrolytic, oxidative, and radical (Horsman & Zechel, 2017). The hydrolytic mechanism specifically acts on Phn that is bound to a carbonyl beta group, by three types of hydrolases. The oxidative pathway uses oxygen and iron represented by two oxygenases, where one of them can be restricted to certain phosphonates. And, the radical process performed by an enzymatic complex lacking specificity, forming a free electron radical (Horsman & Zechel, 2017).

The search for alternative P sources starts when Pi is scarce, activating the global regulation mechanism called Pho regulon (Stosiek & Klimek-ochab, 2019). The Pho regulon stimulates the transcription of enzymes that can metabolize organic molecules so the microorganisms can explore other sources of P (Stosiek & Klimek-ochab, 2019). One of the components of this system is the *phn* gene cluster, or 'C-P lyase' cluster, responsible for the radical mechanisms for phosphonate degradation (Horsman & Zechel, 2017).

The *phn* cluster is composed by 14 genes forming two complexes, the first one transcribing transport proteins to bring phosphonates to the intracellular media (*phn*CDE) (Stasi et al., 2019). The second complex (*phn*FGHIJKLMNOP) results in several components that metabolize phosphonates into partial substrates, until the phosphorous structure is assimilated (Ren et al., 2015). Firstly, phosphonates are ATP-converted (*phn*IGHL), followed by an intermediary breakage resulting in inorganic diphosphate (*phn*M) and finally, the breakage of the C-P bond, releasing the functional carbon group (*phn*J) (Seweryn et al., 2015). The *phn*J gene, also known as 'C-P lyase' enzyme, acts chemically destabilizing the carbon-phosphorous bond – therefore, this one was determined as a marker gene for this metabolism (Seweryn et al., 2015). The expression of the complex *phn*GHIJKLM, the so-called 'core' has been reported to be sufficient for cleavage of the C-P bond (Kamat & Raushel, 2015; Ren et al., 2015; Seweryn et al., 2015).

During MPn degradation, breakage of the C-P bond releases phosphate (PO_4^{-3}) to be consumed by the micro-organisms, and the methyl-group is released as methane (CH_4) to the atmosphere (Karl et al., 2008; Teikari et al., 2018). This methane production through MPn

demethylation is one of the OMP previously well-described, and it has been correlated to the increase of methane concentration in oxygenated waters, including wetlands (Bridgham *et al.*, 2013; Yao, Henny and Maresca, 2016; Teikari *et al.*, 2018). The amount of information on MPn demethylation and methane emissions has been increasing and clarifying more the conditions necessary for it to happen and which are the main groups responsible for it.

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2. CHAPTER 1: OVERVIEW OF OXIC METHANE PRODUCTION FROM CYANOBACTERIA ON SODA LAKES

Abstract

Novel methane (CH_4) production pathways have been recently reported for bacteria revealing the contribution of other organisms to CH_4 accumulation in oxic waters. Gene sequences of methylphosphonate degrading enzymes, one of the major alternative processes, can be used as markers to track them in the environment. In this study, bioinformatic analysis was carried out on metagenomic data from four Pantanal lakes differing in physical and chemical variables to evaluate the potential of bacteria contributing to greenhouse gas emissions. The presence of genes related to four potential oxic CH_4 production processes (*phnJ*, *dmd*, *bch*, *mtt*, and *chl*) were tested, and *mcrA* was studied as a marker for archaeal methanogenesis. We found most genes present in the lakes, although differently distributed among them. *mcrA* gene abundance was low in the water metadata, and the phosphonate demethylation pathway was the most abundant in the lakes with cyanobacterial blooms. Our results corroborated with literature on the higher amounts of dissolved methane and the presence of blooms. When putting all the metadata together, we understood that many possible involvements of cyanobacteria as an OMP contributor. Although, if conditional weight of its impacts.

Keywords: Metagenomics; Multivariate Statistics; Dissolved Methane; Marker Genes

2.1. Introduction

Biogenic methane production was recently demystified from being exclusively carried-out by archaeal organisms to have many other contributors in aquatic systems. The oxic methane production (OMP) pathways are granting new sources, for instance by primary producers' activities like algae and cyanobacteria, which seemed to be enhancing the productions from epi-pelagic regions in oceanic and freshwater environments to the atmosphere. However, to investigate all alternative methane sources of biological origin and determine their climatic feedbacks pose great challenges for climate science.

The new perspectives on biogenic methane production require more information on OMP processes and sources from a plethora of different environments. Even though many micro- and mesocosms studies provide strong evidence of several mechanisms from many organisms releasing methane, expanding them to natural conditions is never an easy task. Likewise, to identify the origin of methane accumulations in oxic environments stem and which are the major sources needs to consider numerous variables which substantially increases the complexity of data analysis.

With that in mind, methane production studies need to account for biological, chemical and physical measures, aside of geographical and meteorological information to draw the "big picture". Methane emissions are the result from a shift in the balance between methane production, oxidation and transportation, varying according to fluctuations in temperature, salinity, pH, organic matter quality, soil type, vegetation, system dimensions, hydrology, pluviosity, climatic conditions, etc. All

these factors combined determine the microbial community composition and structure and, thus, help to cover knowledge gaps addressed in this study.

In this chapter, we performed a general analysis of OMP pathways in the Pantanal soda lakes. As cyanobacteria – to our knowledge - are relevant contributors to OMP, we directed our search on their potential influence on OMP processes. Coupling metagenomics markers described for cyanobacterial processes involved in OMP, and chemical variables from four distinct soda lakes, we explored the major sources and processes of methane and its dynamics in these lakes.

2.2. Methodology

2.2.1. Field survey

Our study objects were the soda lakes from Pantanal's sub-region denominated Nhecolandia (Aquidauana, Mato Grosso do Sul State, Brazil) (Appendix A). The sampling period was during a a partly flooded season, in September 2019, when the water levels was still above of is . We investigated: two eutrophic turbid lakes (04SR and 08SR) with cyanobacterial blooms composed of *Anabaenopsis elenkinii* and *Arthrospira platensis* (Pellegrinetti et al., 2022); one oligotrophic turbid lake (06SR) with a dark color due to elevated amounts of humic compounds; and lake 07SR characterized as a non-saline clear vegetated oligotrophic lake with crystalline water. In Appendix C, the meteorological parameters since 2017 are shown.

2.2.2. Metagenomics shotgun sequencing

The water samples collected and the metagenomics dataset were obtained in the study conducted by Pellegrinetti et al (2022) and are detailed as follow. Water samples were collected from the lakes at 20 cm depth and preserved in liquid nitrogen for their transport to the lab at CENA/USP where they were stored at -80°C until lab analyses. In the lab, samples were lyophilized and resuspended in DNA buffer. DNA extraction was performed using the PowerLyzer PowerSoil Isolation kit (MoBio Laboratories Inc., CA, USA), following the manufacturer's protocol. The quality of the extraction product was evaluated by electrophoresis using a 1% agarose gel in 1x TBE buffer (400 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA), 1-3 µl of DNA and 2 µl loading buffer, which increases the sample density with the GelRed™ fluorescence dye. Final concentrations were verified using a quantitative fluorometry device, i.e. Qubit® 2.0 Fluorometer (Life Technologies™).

After DNA purification, the products were forwarded to the Centralized Laboratory for Multiuser of Functional Genomics Applied to Agriculture and Agroenergy (ESALQ/USP) for final processing and sequencing on a Illumina HiSeq platform (Illumina, Inc. USA) using the kit HiSeq

Reagent Kit V3. A metagenomics library was prepared using the Nextera XT DNA Sample Preparation kit (Illuminas, Inc., USA), following the manufacturer's protocol. Using quality graphics on FastQC 0.10.1 (bioinformatics.babraham.ac.uk/projects/fastqc/) allowed us to check for sequence quality. Paired fragments read overlapping was verified together with consensus sequences generated via the PEAR software (Pair-End reAd mergeR) (Jiajie Zhang et al., 2014). Sequences with a quality lower than 20 Phred (average error) and fragments with less than 50 bp were removed using the filter Seqyclean 1.3.12 (Zhbannikov et al., 2017).

2.2.3. Metagenomics analysis

Bioinformatics analysis of the metagenomics dataset was performed using specific approaches for detecting the pathway we were interested in. The database used to annotate the whole metagenome was the nr-NCBI (non-redundant RefSeq). The metagenomics data was aligned with database using the function `blastx` at the DIAMOND protein aligner tool (Buchfink et al., 2014). Using the metagenome analysis software MEtaGenome ANalyzer 6 (MEGAN) (Huson et al., 2016), annotations were done via the LCA algorithm (Beier et al., 2017). Functional classification was placed into the KEGG database, using the orthologues database for determining the molecular functions (KO) for each enzyme we have analyzed (Kanehisa et al., 2016).

2.2.4. Dissolved methane quantification

The dissolved gas datas used in this study was collected and processed by the Ph.D. candidate Yara Barros Feitosa and is detailed as follows. Dissolved gas concentrations in the water were analyzed via the headspace method during *in situ* sampling. Three statistical replicates were sampled from four different points in the lake for this analysis. Using 140 ml polypropylene syringes, 105 ml of water were collected from 5-10 cm water depth, following a proportion air:water of 1:3 of the total syringe volume. Immediately after sampling, the syringes were manually agitated for one minute, followed by the injection of headspace air in 20 ml vials properly sealed. The samples were analyzed using a Shimadzu GC-2014 gas chromatograph (Shimadzu Co., Columbia, MD, EUA), located at the Laboratory of Cellular and Molecular Biology (CENA/USP). For methane calculations, the data was corrected using Henry's Law for headspace-water partitioning of the gas. Afterwards, the dissolved gas concentrations in the water were estimated from the corrected concentrations, using the Ideal Gas Law:

$$P'V = nRT$$

P'= partial pressure methane in the atmosphere;

V= volume, in mL;

n= moles of methane;

R= gas constant (0.0821 atm L mol^{-1} K^{-1});

T= temperature (25 ºC)

2.2.5. Statistical analysis

In order to analyze multiple OMP pathways, metagenomics data were normalized and compared using the function `compare` of MEGAN6. After total hits of each sample were compared marker genes of interest were selected from the database for specific annotation (Table 1). The hits retrieved were normalized and the relative abundance was presented as a heatmap, with a Z-score for scaling of significance evaluation, hierarchical clustering and a dendrogram created based on similarity. The heatmap was performed with the function `heatmap.2()` from the package `gplot()` in R (*https://cran.r-project.org/web/packages/gplots/index.html*).

Table 1. Reference gene markers for oxic methane production (OMP) used for screening the metagenomic data of the studied soda lakes.

Methane Production	**Marker**	**Gene**	**Enzyme**	**Function**
Oxic methane production (OMP)	bch	*bch*X, *bch*Y, *bch*Z	Chlorophyllide a reductase (COR)	Reduction of bacteriochlorophyll
	chl	*chl*L, *chl*N, *chl*B	Ferredoxin:photochlorophyllide reductase (DPOR)	Reduction of chlorophyllide
	dmd	*dmd*A,*dmd*B, *dmd*C, *dmd*D	Dimethylsufoniopropionate demethylase complex	DMSP demethylation
	mtt	*mtt*B, *mtt*C	Trimethylamine---corrinoid protein Co-methyltransferase	Trimethylamine demethylation
	phn	*phn*G, *phn*H, *phn*I, *phn*J, *phn*L, *phn*M	C-P lyase 'core'	Methylphosphonate demethylation
Classical archaeral methanogenesis	MCR	*mcr*A-*α,* *mcr*A-*β,* *mcr*A-*γ*	Methyl-coenzyme M reductase	Methane from acetate, hydrogen or methyl compounds

Dissolved methane concentrations were analyzed by Analysis of Variance (ANOVA), $p < 0.05$ significance level, and Tukey's post-hoc using the package `agricolae()` (*CRAN.R-*

project.org/package=agricolae), and the boxplot were generate using the package `ggplot2()` (*CRAN.R-project.org/web/packages/ggplot2/index.html*).

To evaluate the main factors directly related to OMP or as a driver determining the potential of the genes in the soda lakes, multivariate statistic of Principal Component Analysis (PCA) was performed, using the packages `FactoMineR()` (*https://cran.r-project.org/web/packages/FactoMineR/index.html*) and `factoextra()` (*https://cran.r-project.org/web/packages/factoextra/index.html*). The data was scaled as a logarithmic matrix to avoid null values, and vectors' distances were calculated based on Euclidian distance. Chemical variables used for this analysis are given in (Appendix B), as average and standard deviation values. All statistical analyses were performed using the software R 4.0.5 (R Core Team, 2020).

2.3. Results

2.3.1. Comparative abundance of OMP metagenomics markers

A normalized comparison for the relative abundance of hits corresponding to methane production (*phn, dmd, bch, chl* and *MCR*) markers was performed using the KEGG database (Fig. 2). In total, 20,301,228 reads were normalized from which 2,537,976 reads could be assigned in the metagenomic comparative analysis when using the BlastX function. The total of reads for each sample are shown in Appendix D. We used the average hits for each enzyme potentially able to generate methane. It is important to keep in mind that with our heatmap analysis we cannot compare genes, but only samples.

Starting with the classical methanogenesis, *mcr*A hits were more abundant at lake 07SR, a non-saline lake without any cyanobacteria or algae blooms. Because this metadata corresponds to only oxic water samples, not much archaea can be expected, specially methanogenics ones – as this is a strictly anaerobic process.

For demethylation pathways of organic matter, we focused on methyl-phosphonate (MPn), trimethylamine (TMA), and demethylsuphoniopropionate (DMSP), due to their previously shown relationship with cyanobacteria (Table 2). MPn demethylation by the *phn* cluster or the 'C-P lyase' was mostly present in lakes with cyanobacterial blooms, with the highest abundance in lake 04SR. Demethylation of TMA occurred via a set of methyltransferases (*mtt*) and of DMSP via a complex set of demethylases. Both genes had a similar distribution pattern with a higher abundance in lake 06SR. In contrast to MPn degradation, *mtt* and *dmd* clusters were absent or in very low abundance in the lakes with algal blooms. This suggests differences in their microbial communities and thus roles in each soda lake type.

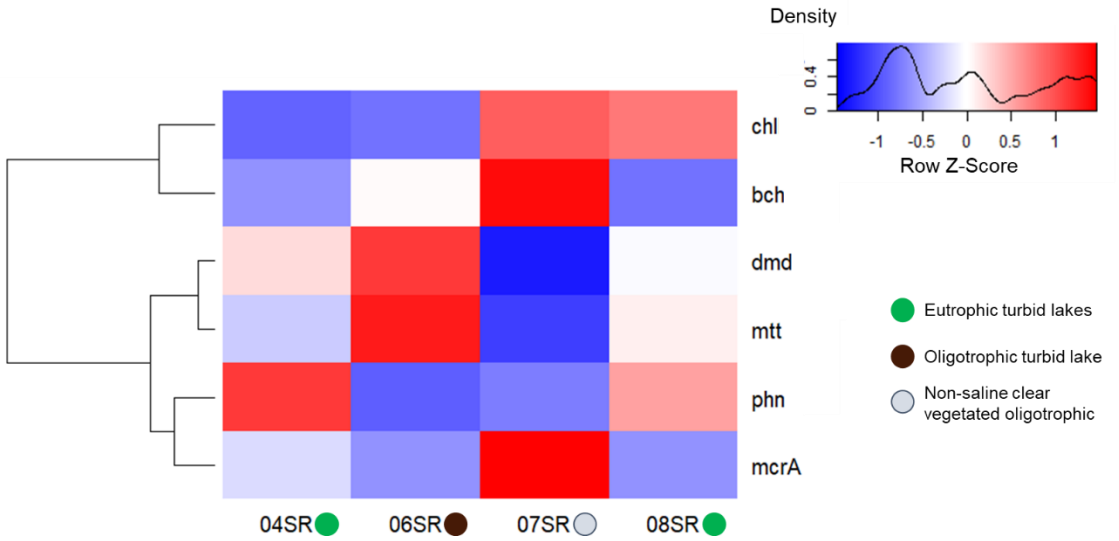

Figure 2. OMP potential distribution among the soda lakes based on similarity of their relative abundance for each lake.

Lastly, bacteriochlorophyll and chlorophyll degradation suggested from phototrophic microorganisms were represented by the bch and chl clusters, respectively. The heatmap shows that bch complex is more abundant in lake 07SR and absent in lake 06SR. Meanwhile, chl cluster was not only present in lakes 07SR and 08SR. There is no clear pattern with lake type and communities harboring these genes.

2.3.2. Methane concentrations in the studied soda lakes

Using the headspace method, methane concentrations were quantified in order to identify if there is any difference in methane production according to lake type. Mean values and standard deviations for dissolved methane concentrations are presented in Appendix E. There was a significant difference in methane concentrations between lakes, (ANOVA, $F=122.5$; $p < 2e^{-16}$) as demonstrated below. The boxplot shows that lakes 04SR and 08SR have the highest methane values (Fig. 3). Hence, the soda lakes with algal blooms are characterized by more dissolved methane in the water than the oligotrophic lakes 06SR and 07SR. Lake 06SR has insignificant amounts of this gas during the evaluated period when compared to the other lakes.

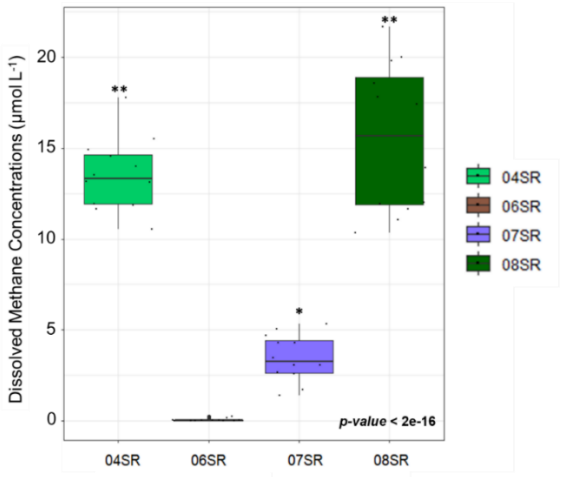

Figure 3. Dissolved methane concentrations (µmol L-1) from the soda lakes. Statistical differences with significant for $p < 0.05$. Statistical significance is shown as (**) higher average; (*) intermediary average.

2.3.3. Multivariate analysis of OMP chemical and microbial variables to lake methane

The variation between lake samples along PC1 axis is 50.6%, and along PC2 35.5% indicating more differences between lakes across axis 1 (Fig. 4). For the biplot, we chose some important factors that are relevant to the studied OMP pathways. The chemical variables selected were: chlorophyll *a* (Chla), dissolved organic carbon (DOC), dissolved methane concentrations (CH4), dissolved oxygen levels (DO), nitrate (NO3) and ortho-phosphate (OPO) concentrations.

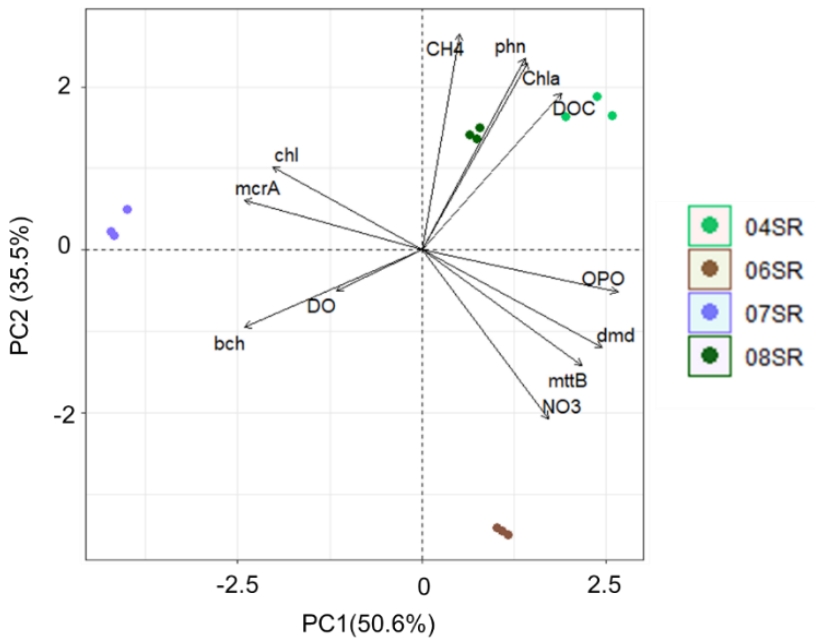

Figure 4. Principal component analysis (PCA) of environmental variables and abundance of OMPmarker genes.

The soda lakes presenting cyanobacterial blooms (in green) were closely related based on the variables we chose (Fig. 4). In contrast, lakes 06SR (brown) and 07SR (light blue) were completed separated from the eutrophic lakes and from each other, demonstrating their dissimilarity in environmental variables. For the bloom-presenting lakes the determinant chemical parameters were dissolved CH_4, DOC, and Chla, and the phn is most abundant genetic marker for OMP compared to all other lakes. For lake 06SR, nitrate and ortho-phosphate concentrations were more influential, together with a considerable abundance of dmd and mtt OMP marker genes. Lastly, in lake 07SR the *mcr*A marker gene for classical methanogenesis was prevalent, indicating that the methane there possibly comes mostly from archaea activity or plant-mediated transported residuals, together with chl for chlorophyllide degradation and bchl for bacteriophyll reduction.

2.4. Discussion

In order to explore the potential of oxic methane production pathways in the studied Pantanal soda lakes, we performed a metadata investigation for environmental indicators. Four soda lakes with different physical and chemical characteristics were studied: oligotrophic turbid lake (06SR), clear vegetated oligotrophic lake (07SR), and two eutrophic turbid lakes (04SR and 08SR), during a partially flooded season in September of 2019.

Many OMP processes previously described in the literature for being directly or indirectly conducted by cyanobacteria or, at least, other photosynthetic organisms (Table 2). The interest in cyanobacteria comes from a particular concern when we consider the extent of cyanobacteria blooms potentially massively impacting greenhouse gas emissions from lakes. The review of Bižić (2021) draws attention to the contribution of cyanobacterial blooms from anthropogenic activities to methane emissions either indirectly through the creation of hypoxic dead zones, feeding classical methanogenesis, or their direct methane production via OMP in oxygen saturated waters.

Table 2. Literature survey of mechanisms of potential metabolic pathways for explaining oxic methane production (OMP) in aquatic environments.

Potential processes	**Suggested mechanisms**	**Reference**
Demethylation of organic compounds	Methane as a subproduct from enzymatic bond breakage of R-CH_3	Karl *et al.*, 2008; Damm *et al.*, 2010; Bižić-Ionescu *et al.*, 2018; Teikari *et al.*, 2018
Light-driven primary productivity	Carbon fixation subproducts are used as methane precursors	Bižić *et al.*, 2020
Cyanobacteria-hydrogenotrophic Archaea	Nitrogenase activity generating residual H_2	Berg, Lindblad and Svensson, 2014
Association between Cyanobacteria-Archaea	Cyanobacterial structure creates a micro-anoxic environment for archaea	Grossart *et al.*, 2011; Batista *et al.*, 2019
Non-enzymatic methane formation	Reaction between free radicals release by cyanos	Ernst *et al.*, 2022
Nitrogenase-like enzyme activity	Fe-only nitrogenase can reduce CO_2 to CH_4 during N-fixation	North *et al.*, 2020
Emissions from plants; fungi, algae	Breakdown of organic compounds by reactive oxygen species (ROS)	Wang *et al.*, 2013; Lenhart *et al.*, 2015
Chlorophyllide reduction	Its degradation can release methane precursors, or the COR enzyme might catalyze CO_2	Perez-Coronel, Hart and Beman, 2021

The soda lakes that are classified as eutrophic turbid lakes presented cyanobacterial blooms in parallel to higher concentrations of methane dissolved in the water column (Fig. 3). Followed by the oligotrophic lakes 07SR and 06SR, with a relevant reduction in phytoplankton productivity and dissolved methane. These results are similar to what was previously reported for the Pantanal soda lakes from the 'Centenário Farmland' area (Barbiero et al., 2018). In this study, the authors demonstrate that not only the presence but also the intensity of cyanobacterial blooms interferes with the methane production rates.

Albeit, the only marker gene we found on the metagenome to be highly abundant in lakes with cyanobacteria blooms is the phn cluster for MPn demethylation. We used the genes representing the core cluster of the 'C-P lyase' enzyme, the ones that actively act on the breakage of the carbon-phosphorous bond of phosphonates. The C-P lyase cleaves the carbon and phosphorous bond, releasing the C group as methane and thereby making P available for microbial usage (Ruttenberg & Dyhrman, 2012; Stosiek & Klimek-ochab, 2019).

It is described that these soda lakes are considerably drawn for phosphorous, especially lake 08SR (Appendix B), which renders this environment more conducive to the consumption of alternative sources of organic phosphorous. Figure 4 also shows a negative relationship between the

bloom-harboring lakes and orthophosphate concentrations – which reinforces the lower abundance of this nutrient there. This pathway mainly occurs in environments presenting P scarcity and serve as an explanation for CH_4 accumulation in oxygen-rich or even oxygen supersaturated surface waters (Repeta et al., 2016). Literature commonly refers to the presence of the C-P lyase genes of Proteobacteria, Actinobacteria, Cyanobacteria, and Firmicutes representatives (Villarreal-chiu et al., 2012; Wang et al., 2017). Therefore, this would be a very interesting pathway to be explored in more details in the soda lakes to evaluate the cyanobacterial contribution to total methane production rates.

On the other hand, the other two OMP pathways via demethylation of organic compounds, for DMSP and TMA were similarly distributed in high abundance in lake 06SR (Fig. 2). DMSP is a methylated compound known to be produced by phytoplankton, and its demethylation can provide energy and carbon sources for heterotrophic bacteria (Althoff et al., 2014; Damm et al., 2010). Andreote *et al.* (2018) identified the dmd genes corresponding to Actinobacteria, Betaproteobacteria and Cyanobacteria in an oligotrophic turbid lake similar to 06SR from another area of the Brazilian Pantanal.

TMA is a common subproduct from organic matter degradation, largely present in hypersaline lakes, which can be used as methane precursor under anoxic conditions as well as in oxygen supersaturated regions (Bižić-Ionescu et al., 2018; McGenity & Sorokin, 2018). It is interesting that the PCA analysis shows a close relation of nitrate and orthophosphate in lake 06SR with the abundance of TMA demethylases (Fig. 4). The elevated presence of nitrogenated compounds can indicate the presence of more complex structures, such as TMA, that are degraded and released in the water. Therefore, this indicates the pathway's potential contribution to some other dependent processes.

The results show that genes for the reduction of chlorophyll and bacteriochlorophyll do not show a distribution pattern among the studied lakes. They mainly occurred in lake 07SR, quite influenced by the levels of dissolved oxygen (Fig. 4). We could attribute the abundance to the presence of non-oxygenic phototrophic bacteria that might dominate in this environment, as cyanobacteria are not present to compete in this role. Although, *chl* were also very abundant in lake 07SR, this set is also highly present in the eutrophic lake 08SR. For the last one, it is likely to be related to the cyanobacterial bloom. However, we cannot conclude this inference as lake 04SR with a high cyanobacterial abundance showed a very low abundance of this marker gene.

The hypothesis raised in Perez-Coronel & Beman (2021) is that (bacterio)chlorophyll methoxyl groups can serve as precursors of methane, or these enzymes directly catalyze methane production. The *bch* gene corresponds to chlorophyllide a reductase (COR) that acts on chlorin during bacteriochlorophyll biosynthesis (Lockhart et al., 1996; Perez Coronel, 2020). The *chl* set of genes is often found in photosynthetic bacteria corresponding to ferredoxin:protochlorophyllide reductase (DPOR) that reduces protochlorophyllide (Lockhart et al., 1996; Perez Coronel, 2020). Although it is

important to emphasize that bch and chl are quite ordinary genes in many organisms, so we were not able to clearly understand their acting as an OMP on the soda lakes.

We do understand that there are weighty variables that need to be accounted before carefully doing any statements on OMP. For instance, the relationship found here and other literature on cyanobacterial blooms and large methane productions cannot be referred exclusive to their active involvement. At the end of blooms, cyanobacteria sink to the bottom of the water column and can be used as source for archaeal methane production via the methyl coenzyme M reductase, or MCR. The classical methanogenesis performed by archaea in the sediment of lakes uses products generated by organic matter degradation. Thus, cyanobacteria can be regarded as food for the classical methanogenic pathway in the sediment – an indirect association to archaea (Fazi et al., 2021; León-Palmero et al., 2020; Yan et al., 2019; Zhou et al., 2020). Because we have only analyzed water metadata, yet, we cannot say much about this relationship in sediments. The strong influence of DOC on lakes 04SR and 08SR in the water column (Fig. 4) might indeed reflect an autochthonous origin of methane, but more detailed studies are needed for more conclusive statements.

Another relevant point to be considered for the studied soda lakes is their physical characteristics, such as depth and dimensions. The soda lakes are very shallow (2 m of depth, at most) compared to the other environments where OMP has been reported. The short distance between sediment and surface can cover up the presence of methane and oxygen accumulation in specific layers (Bogard et al., 2014; Deemer et al., 2016). Bastviken *et al.*, 2010 consider that the physical characteristics of bathymetry, mixing, turbulence, and hydrostatic pressure are the main responsible for stimulating the releasing of methane from the Pantanal lakes. Therefore, the hydrostatic pressure would be lower, triggering methane ebullition from the sediments.

Moreover, the lakes' dimensions favor the lateral transport from the sediment and vegetation on lakeshore. Lateral transport from the littoral zones is commonly pointed as a relevant source of methane in the epilimnion of some aquatic systems (Bastviken et al., 2010; León-Palmero et al., 2020). Many red flags were brought as well when point to OMP as a major source alone when considering experimental extrapolations as some variables cannot be accounted on calculations (Peeters et al., 2019). DelSontro et al. (2018) developed a model to understand the physical factors, including effects of lateral transportation for methane emissions. Yet, they identified that the pelagic OMP greatly contributes to methane emission - also in shallow lakes. Thus, they provide evidence to combine the various mechanisms and controlling factors when evaluating lake methane production mechanisms.

Anyhow, our intentions were to evaluate whether cyanobacteria or other phototrophic organism are important for methane production in the soda lakes. Beaulieu et al. (2019) corroborated the occurrence of OMP jointly with considerable chlorophyll *a* concentrations, hence primary productivity. Donis *et al.*, 2017 included many variables on their calculations for methane accumulations at Lake Hallwil (Switzerland), and still their results showed that the gas was originated

from surficial layers under oxic conditions. Similarly, coexisting with phytoplanktonic activity, Günthel *et al.*, 2019 also concluded that OMP can happen in the upper oxic, pelagic water layer of Lake Stechlin (Germany). If such connections are in fact occurring, there are many other methane sources to be considered them it was previously considered and they might need as much attention as well.

We consider that this kind of investigation can help on determining methane dynamics and their underlying processes which may greatly vary in different environments. When analyzing the metadata together with the physical and chemical parameters, we were able to better explain the potential of different methane formation pathways in the soda lakes. These initial results pointed that this approach represents a very interesting way to determining future developments in methane production, as until now it's not possible to specify which methane production pathway is dominating. In the next chapters, we will deepen the investigations about two specific OMP pathways related to cyanobacteria blooms and the Pantanal soda lakes.

2.5. Conclusion

With this study we conclude that:

- The Pantanal soda lakes indicate mechanisms for many possible pathways of oxic methane production, indeed related to cyanobacteria;
- Soda lakes with cyanobacterial blooms show the highest methane concentrations in the water, and a high potential for the demethylation of organic phosphorous which may greatly account for the measured CH_4 productions;
- Although physical and chemical parameters of the soda lakes indicate many different methane formation processes, our metadata analysis provided deeper insights into the potential role of OMP and in particular cyanobacteria.

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3. CHAPTER 2: PHOSPHONATE CONSUMERS POTENTIALLY CONTRIBUTING TO METHANE PRODUCTIONS IN BRAZILIAN SODA LAKES

Abstract

'C-P lyase' pathway corresponds to phosphonates degradation that can lead to methane release in oligotrophic lakes, where the carbon-phosphorous bond is enzymatically broken for microbial consumption of phosphate in events of phosphorous starvation. Cyanobacteria have been established as an important player in the C-P lyase pathway, contributing to OMP in lakes and oceans. Here, we investigated five soda lakes from the Brazilian Pantanal in two contrasting seasons (dry and wet) for methane production through C-P lyase metabolism. We applied bioinformatic tools to analyze metagenomic data taxonomically and functionally for phosphonate catabolism, in order to evaluate the potential of methane production in the lakes. Also, we annotated genomes and Metagenome-Assembled Genomes (MAGs) of cyanobacteria to identify more precisely which members of this phylum are potentially involved in this process. As result, we identified a relevant change in the microbial community between seasons, with a high abundance of the 'C-P lyase' gene related to Cyanobacteria. The MAG assigned as *Raphidiopsis* sp. was the main cyanobacterium harboring the C-P lyase gene cluster, which corroborated with our previous findings for this genus, leading us to hypothesize that the C-P lyase pathway is part of its adaptation to survive. Seasonal pluviosity together with orthophosphate, nitrate, and iron concentrations are very important drivers for this metabolism and demonstrated that two lakes with blooms have the proper conditions for it. We demonstrated the potential for the 'C-P lyase' pathway to occur on the soda lakes and consequently acting as OMP to be relevant. Althought, there is no direct relation to the cyanobacterial blooms, this pathway is likely adding enough to the final methane emissions.

Keywords: C-P lyase; Ortho-phosphonate; Cyanobacterial blooms; Metagenomic-Assembled Genome

3.1. Introduction

Microbial methyl-phosphonate (MPn) degradation in oxygen oversaturated waters has raised the attention for the release of methane (CH_4) as a byproduct. Also called the 'C-P lyase' pathway, MPn demethylation can occur when the enzyme C-P lyase hydrolyses the stable bond between carbon and phosphorous. In this pathway, the phosphate group is used by microbes and incorporated in their cellular structure, and the methyl group is released into the environment.

Recently, many studies have shown a broad potential of Cyanobacteria to release methane through 'C-P lyase' pathway. As demonstrated in Chapter 1, this can be a relevant OMP process to be explored in the Pantanal soda lakes, in particular in those with intensive cyanobacterial blooms. Therefore, these productions may contribute considerably to the lake's greenhouse gas emissions and lead to methane accumulations in the oxic water column during bloom formation (Karl *et al.*, 2008; Adams *et al.*, 2008; Gomez-Garcia *et al.*, 2010; Teikari *et al.*, 2018; Khatun *et al.*, 2020)

Even when anaerobic methanogenesis is high in the sediments, substantial oxygen production by cyanobacteria blooms, theoretically, counteract high methane concentrations via

oxidation in surface waters. Thus, alternative processes for methane production may occur in these environments, leading to substantial methane accumulations. The alternative 'C-P lyase' pathway holds an interesting potential to occur in the Brazilian Pantanal soda lakes, especially in those providing proper conditions for cyanobacterial mass blooms.

In this chapter, we aim to investigate the microbial community related to OMP through MPn demethylation, with special attention to cyanobacterial groups and their interactions with others carrying the *phn*J gene set. Environmental factors such as seasonality, nutrient availability, and pluviosity are relevant drivers controlling the potential of this pathway. We believe that cyanobacteria use the phosphorus of MPn under the P-limiting conditions of the Pantanal soda lakes, contributing to the high methane concentrations previously described in these environments, while competing for alternative sources of phosphorous.

3.2. Methodology

3.2.1. Field survey

For this study, we performed a temporal comparison analysis. In total, five soda lakes from the Pantanal's sub-region denominated Nhecolândia (Aquidauana, Mato Grosso do Sul State, Brazil) were investigated (Appendix A). Sampling periods chosen represented a a dry season (October/2017), and a partly flooded season (September/2019). The chosen environments comprise three eutrophic turbid lakes (04SR, 08SR, and 09SR) with cyanobacterial blooms in both periods; one oligotrophic turbid lake (06SR) with dark color and elevated amounts of humic compounds; and lastly, lake 07SR, which is characterized as a non-saline clear vegetated oligotrophic lake with crystalline water (Appendix B and C).

3.2.2. Bioinformatic analysis on metagenomics data

After sequencing and data preparation as described in the previous chapter, in this study, we performed a more specific bioinformatics approach to analyze the pathway of interest. Thus, we created a reference database of amino acid sequences for the core of the C-P lyase enzyme (PhnJ). The database was aligned with metagenomics data using the function `blastx` of the DIAMOND protein aligner tool. Using the metagenome analysis software MEGAN6, taxonomic annotations were done using the LCA algorithm, which assigns reads to the lowest common ancestor in the NCBI taxonomy, using the following setting parameters for LCA naive: min. score 50.0, max. expected 0.01, min. percent identity 50, min. support percent 0.05, top percent 10, and LCA coverage 100%. The core biome was computed using 80.0 as the sample threshold and 1.0 as the class threshold. Contigs

considered to result in an uncertain classification was described by the software as 'Unassigned'. Functional classification was placed into SEED database, using the best alignment available for the C-P lyase pathway (Beier et al., 2017).

Microbiome profile data was extracted from MEGAN6 and explored for determination of microbial community structure and composition. Alpha- and beta-diversity, including richness estimators, diversity index and the multivariate analysis based on Bray-Curtis distance and nMDS (ordination non-metric multidimensional scaling), were calculated using the package `phyloseq()` (McMurdie & Holmes, 2013) statistical program R 4.0.5 (R Core Team, 2020). Statistical analysis with Tukey's test HDS, analysis of variance (ANOVA) and permutational analysis of variance (PERMANOVA) were performed using agricolae`()` (*CRAN.R-project.org/package=agricolae*). Regression model and Pearson correlation were analyzed in PAST: Paleontological Statistics software version 2.17c (Hammer et al., 2001). Visual graphics were generated with the package `ggplot2()` in R (*CRAN.R-project.org/web/packages/ggplot2/index.html*).

3.2.3. Amplicon and genomic screening for phnJ gene

Two approaches were used to refine the identification of cyanobacteria contribution to MPn demethylation: PCR amplification and genomic annotation. Firstly, we selected strains isolated from the saline/alkaline waters at the Pantanal subregion's Nhecolândia to perform an amplification reaction of the *phn*J gene (Table 3). The strain *Nodularia spumigena* CENA596 was used as a positive control because previous genome screening indicated the presence of the C-P lyase cluster. DNA extraction was performed using the PowerSoil® DNA Isolation Kit following the protocol instructions (MoBio Laboratories, Inc., CA, USA). For the PCR amplification reaction, we used the primers set, *phn*JF (TTCTAGGGCGTGCATTTTGC) and *phn*JR (ACCAACGCCGTGAATATTCG), at the following conditions: 3 min at 94°C; 30 cycles of 94ºC for 30s, 60.5ºC for 30s, and 72ºC for 3 min; and a final elongation at 72ºC for 10 min (Teikari et al., 2018). All the strains are part of the cyanobacterial collection from the Laboratory of Cellular and Molecular Biology located at CENA/USP, coordinated by Prof. Dr. Marli Fiore.

Table 3. Cyanobacterial strains isolated from the Brazilian Pantanal soda lakes used for *phnJ* amplicon screening.

Classification	Strain ID	Soda lake
Allinostoc sp.	CENA511	Salina Verde
Alkalinema pantanalinense	CENA528	Salina Preta, Centenário
Cyanobacterium sp.	CENA527	Salina Grande
Geminocystis sp.	CENA526	Salina Centenário
Leptolyngbia sp.	CENA520	Salina Verde
Arthrospira sp.	CENA597	Salina Grande
Microchaete sp.	CENA541	Salina Verde/Vermelha
Nososilinea sp.	CENA512	Salina Verde
Nostoc sp.	CENA547	Salina 67 mil I
Pantanalinema rosaneae	CENA516	Salina Verde
Phormidium sp.	CENA525	Salina Centenário
Pseudoanabaenaceae	CENA510	Salina Verde, Tradagem
Tolypothrix sp.	CENA550	Salina 67 mil I

Meanwhile, using the Blast (Basic Local Alignment Search Tool) tool, screening of the genomic data was also performed based on a nucleotide database created for the *phn* gene cluster. Table 4 provide genomes and metagenome-assembled genomes (MAG) correspondent to cyanobacteria strains recovered from the soda lakes. These genomes were sequenced and assembled by the Ph.D. candidate Endrews Delbaje as well as the assemblage of the MAGs. As no pre-curated database for the gene cluster was available, manual curation on available data in the open-access database from GenBank NCBI was required to compose our reference database for *phnJ* (ncbi.nlm.nih.gov/genbank/). Ordination and visualization of the complete *phn* gene cluster was analyzed using the Artemis software (Carver et al., 2012).

Afterwards, phylogenetic analysis of cyanobacterial sequences was done aiming to identify relationships between the cyanobacterial lineages that carry the C-P lyase gene and the identification of the matching MAGs, with data available from the NCBI GenBank. Phylogenetic trees were constructed using the Maximum Likelihood method of the Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al., 2018), with the best evolutive model determined by the ProtTest 3.4.2 tool (Darriba et al., 2017) to generate a bootstrap consensus tree inferred from 1000 replicates.

Table 4. Genome and Metagenome Assembled-Genome (MAG) data description from cyanobacteria strains retrieved from the studied Brazilian Pantanal soda lakes.

Genome						
Family	**Genus/Species**	**Strain ID**	**Soda lake**	**Scaffolds**		
Leptolyngbyaceae	*Alkalinema pantanalinense*	CENA528	Salina Preta, Centenário	1		
Nostocales	*Anabaenopsis elenkinii*	CCIBt3563	Salina do meio	1		
Chroococcaceae	*Geminocystis* sp.	CENA526	Salina Centenário	232		
Oscilatorialles	*Arthrospira platensis*	CENA597	Salina grande	1		
		CENA650	08SR	28		
Leptolyngbyaceae	*Pantanalinema rosaneae*	CENA516	Salina Verde	1		
Metagenome-Assembled Genome (MAG)						
Family	**Genus**	**Strain ID**	**Soda lake**	**Scaffolds**	**Completeness**	**Contamination**
Nostocaceae	*Nodularia*	Mag_19_01SR_18	01SR	280	91.08	0.00
Phormidiaceae	Unclassified	Mag_11_01SR_18		224	96.65	1.54
Cyanobiaceae	Unclassified	Mag_44_01SR_19		88	98.23	0.95
Phormidesmiaceae	Unclassified	Mag_33_01SR_19		364	77.32	1.63
Phormidiaceae	*Limnospira*	Mag_41_04SR_17	04SR	241	96.07	0.22
Nostocaceae	*Nodularia*	Mag_27_04SR_17		202	92.63	0.00
Phormidiaceae	*Limnospira*	Mag_36_04SR_18		209	97.16	0.00
Nostocacea	*Nodularia*	Mag_42_04SR_18		264	93.11	0.00
Phormidesmiaceae	Unclassified	Mag_14_04SR_18		321	90.40	1.54
Cyanobiaceae	PCC7001	Mag_12_04SR_19		132	97.00	0.54
Nostocaceae	*Nodularia*	Mag_19_04SR_19		356	90.7	0.00
Cyanobiaceae	Unclassified	Mag_48_05SR_18	05SR	262	77.03	2.13
Phormidesmiaceae	Unclassified	Mag_49_05SR_18		340	89.31	1.22
Nostocaceae	*Raphidiopsis*	Mag_34_05SR_19		82	98.04	1.78
Nostocaceae	*Nodularia*	Mag_35_05SR_19		217	88.55	0.00
Cyanobiaceae	PCC7001	Mag_17_06SR_17	06SR	76	93.48	0.27
Cyanobiaceae	PCC7001	Mag_19_06SR_19		160	95.24	0.41
Phormidiaceae	*Limnospira*	Mag_20_08SR_17	08SR	224	94.32	0.00

Nostocaceae	*Raphidiopsis*	Mag_45_08SR_18		96	96.26	0.00
Nostocaceae	*Nodularia*	Mag_18_08SR_18		400	92.05	0.48
Microcystaceae	*Snowella*	Mag_56_08SR_18		440	86.50	0.07
Phormidesmiaceae	Unclassified	Mag_24_08SR_18		239	95.56	1.81
Nostocaceae	*Raphidiopsis*	Mag_7_08SR_19		158	98.3	0.00
Nostocaceae	*Nodularia*	Mag_22_08SR_19		238	90.96	0.24
Phormidesmiaceae	Unclassified	Mag_13_08SR_19		422	75.41	1.40
Nostocaceae	*Raphidiopsis*	Mag_8_09SR_19		40	97.41	0.22
Nostocaceae	Nodularia	Mag_3_09SR_19	09SR	255	88.43	3.37
Unclassified	-	Mag_26_09SR_19		106	92.45	0.00

3.3. Results

3.3.1. C-P lyase gene marker relative abundance

In total, 133,430 reads were analyzed from which 5,196 reads were assigned as phnJ in the metagenomic comparative analysis using the BlastX function. Relative gene abundance was determined for *phn*J between lakes and seasons, given information for inference on the metabolic potential for the C-P lyase pathway. Functional attribution showed a significantly higher abundance of *phn*J during the dry season of 2017 (46.53 ± 23.96) than in the partly flooded period (11.06 ± 10.01), (ANOVA, F=27.96; p=0.00001).

It is noticeable that during 2017 our data showed values two to six times higher than the partly flooded year, in some cases even higher such as 06SR which presented an increase of about 20 times. When considering lakes individually, in both seasons, lakes 08SR e 09SR demonstrated a higher presence of *phn*J; both of them present intensive formation of cyanobacterial blooms (Fig. 5). Lake 04SR, also with a cyanobacterial bloom, accounts for a considerably greater *phn*J occurrence when compared to the oligotrophic ones, 06SR and 07SR.

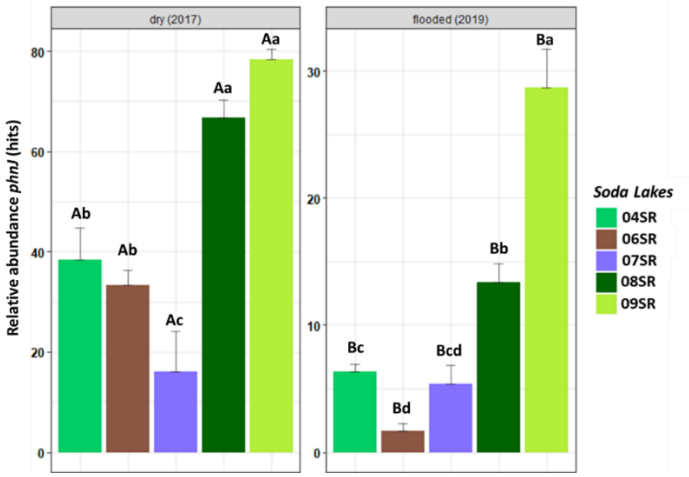

Figure 5. Relative abundance of the marker gene *phn*J on soda lakes during dry and partly flooded seasons. ANOVA, $p < 0.05$. Upper letters represent differences between seasons, the lower letter represents the difference between lakes. Relative abundance is presented in different scales for visual demonstration.

3.3.2. Composition of the microbial community with the potential for phosphonate demethylation

Figure 6 presents the taxonomic classification of the 'C-P lyase' bacterial community on the soda lakes from dry and partly flooded seasons. At the class level, we found that Actinobacteria, Alpha-, Beta-, and Gammaproteobacteria, and Bacilli, compose the core of all lakes and seasons. Despite the lakes' limnological and physico-chemical differences, there is a fluctuation in their abundance when comparing periods, however, no clear dissimilarity of the community composition for this metabolism per se.

During 2017, the dominant classes found were Actinobacteria (7.81-56.18%), Alphaproteobacteria (3.06-26.72%), and Gammaproteobacteria (26.34-41.83%) in all samples. On the other hand, in 2019, we found an alteration in the distribution among the main representatives: with Actinobacteria (15.53-32.09%), Bacilli (44.46-69.74%), and Gammaproteobacteria (6.00-16.38%) remaining in their position, but a significantly higher abundance of Bacilli when compared with the previous period. Betaproteobacteria demonstrated a decrease from the dry season (2.29-8.33%) to this partly flooded period (0.41-5.31%). Other classes demonstrated an abundance lower than 1%.

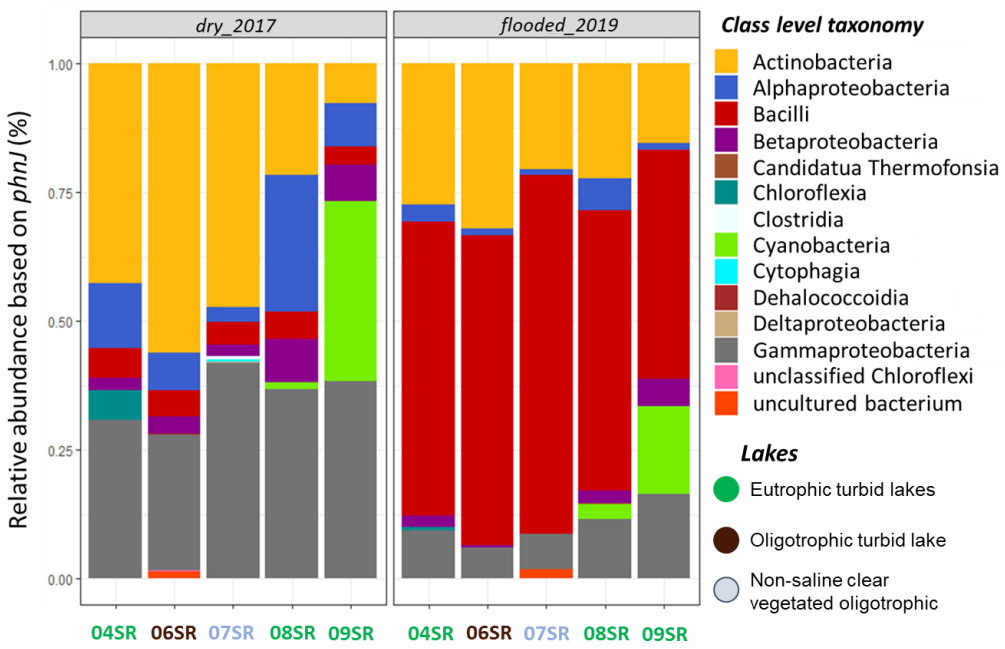

Figure 6. Taxonomic distribution of bacterial community harboring the *phn*J gene for the 'C-P lyase pathway' at the class level.

In respect to the Cyanobacteria participation, lake 09SR includes the presence of Cyanobacteria (35.04%) in dominance as well, distinguishing from the other environments. Discordantly, the others soda lakes with cyanobacterial blooms show none (04SR) or very low (08SR – 1.43%) abundance of this group. On the other hand, lake 04SR considerably presents Chloroflexi in its community, a photosynthetic group of green sulfur bacteria (5.83%). Once more, in 2019, Cyanobacteria kept exclusively showing on lakes 08SR (2.92%) e 09SR (17.02%); however, 04SR does not show any correspondence on photosynthetic organisms. When accessing a deeper taxonomic level for Cyanobacteria, we could identify these matches for the order Nostocales order, family Aphanizomenonacea.

3.3.3. Alpha- and beta-diversity of the 'C-P lyase' community

First, we analyzed the alpha-diversity of the 'C-P lyase' community based on richness estimators ACE, and Shannon's diversity indexes (Table 5). Abundance-based Coverage Estimator (ACE) consider the presence and absence of abundant species. In this study, ACE showed a slight difference among values ranging from 5.33 to 8.87 between lakes (ANOVA; F=3,088; p=0,01), from which the highest richness occurred in lakes 07SR and 08SR in 2017 and 2019, respectively.

Table 5. Richness estimator for each lake in both studied seasons (2017 and 2019). Lower case letters show significative dissimilar means.

Lakes	Richness Estimators
2017	
04SR	$6.00 \pm 1.19_{b}$
06SR	$7.00 \pm 1.25_{ab}$
07SR	$8.87 \pm 1.32_{a}$
08SR	$6.19 \pm 1.23_{ab}$
09SR	$6.00 \pm 1.15_{b}$
2019	
04SR	$5.84 \pm 1.14_{b}$
06SR	$5.67 \pm 1.14_{b}$
07SR	$5.33 \pm 1.04_{b}$
08SR	$6.51 \pm 1.21_{a}$
09SR	$6.0 \pm 1.15_{ab}$

Shannon's measurements accounting for the same weight for rare and abundant taxa, it is highly meaningful for microbial diversity studies (Kim et al., 2017). Nonetheless, Shannon's diversity analysis (ANOVA, F=36.29; $p < 0.05$) revealed higher values for each lake individually during the dry season, especially in soda lakes with blooms (Fig. 7). All lakes with cyanobacterial blooms are more diverse in this analysis, especially lake 09SR showed the greatest index in both periods. As well as lakes 06SR and 07SR are the lowest in this comparison. When comparing seasons, however, it is possible to observe an apparent change, especially in diversity. Shannon's index reinforced dry season (mean value=1,31) having a more diverse bacterial community than flooded season of 2019 (mean value=1,13) (ANOVA, F=6.145; p=0.01) (Fig. 7).

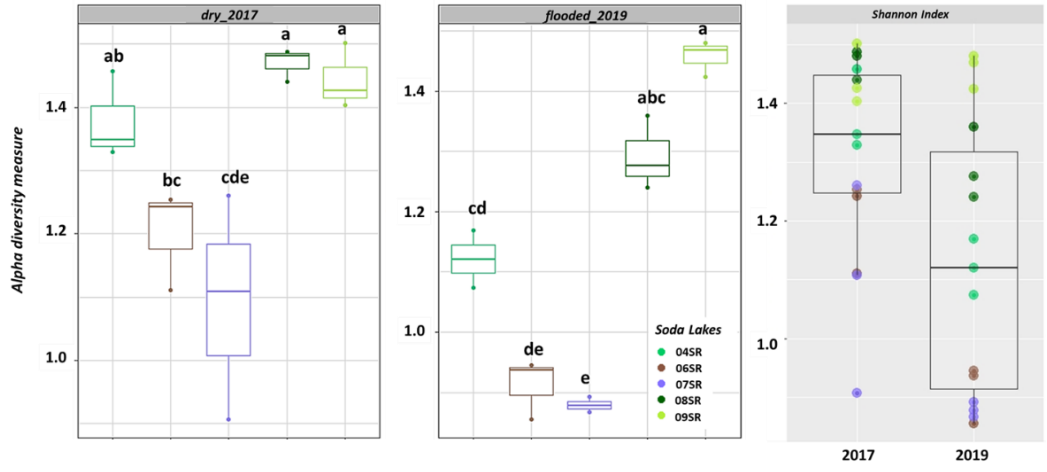

Figure 7. Alpha- and Beta diversity based on Shannon index between lakes and seasons. Letters indicates significant differences among samples, considering $p > 0.05$.

Thereafter, we included the evaluation of the community structure through the studied lakes and seasons. Analysis within seasons allowed us to identify dissimilarities in the microbial communities in 2017 (PERMANOVA; p=0.0001; R=0.9926) and 2019 (PERMANOVA; p=0.0001; R=1). As much as between seasons, where we also indentified divergence for the microorganism diversity responsible for the 'C-P lyase' pathway (PERMANOVA; p=0.0001; R=0.989).

In Figure 8, a non-metric multidimensional scaling (nMDS) analysis was performed to evaluate the dissimilarity between communities. We can see the proximity of lakes with cyanobacterial blooms, and lake 07SR which responds opposite to each other seasonally, and also distant from the other type of lakes studied. We obtained an ordination stress value of 0.07, which is a spatial separation of the data, by type of lake and seasonality, supporting our inferences about the shift on the bacterial community carrying the *phn*J gene.

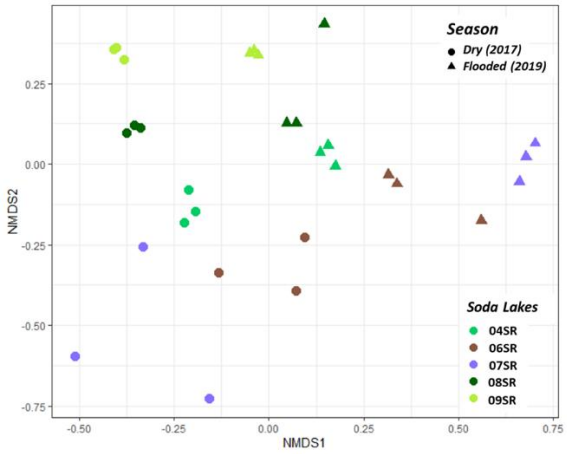

Figure 8. Beta-diversity for 'C-P lyase' bacterial community in two seasons by nMDS dissimilarity analysis. Bray-Curtis as distance matrix. Ordination stress = 0.07317.

3.3.4. Cyanobacteria composing 'C-P lyase' community from the Pantanal soda lakes

We chose PCR amplification and genome/MAG annotation to perform a more complete investigation of which Cyanobacteria from the soda lakes are part of the 'C-P lyase' community. We selected strains isolated from distinct lakes located in the Pantanal subregion, Nhecolândia, including our main interests which are the bloom-forming cyanobacteria genera *Anabaenopsis* and *Arthrospira*. Unfortunately, none of the PCR reactions presented a positive result for any of the strains tested, neither manual nor automatic annotations for the genomic data.

However, we encountered five matches on four MAGs, MAG_45_08SR_18, MAG_34_05SR_19, MAG_7_08SR_19, and MAG_8_09SR_19, retrieved from lakes 05SR, 08SR, and 09SR metagenomes. Figure 9 shows the *phn* cluster found for the soda lake MAGs. All of them are from lakes with cyanobacterial blooms. As Table 4 shows, the matched MAGs are classified as

Raphidiopsis sp., family Aphanizomenonaceae, order Nostocales. This genus is corresponding to the Cyanobacteria composing the 'C-P lyase' community on lakes 08SR and 09SR found on the metagenomic data, as previously shown (Fig. 6).

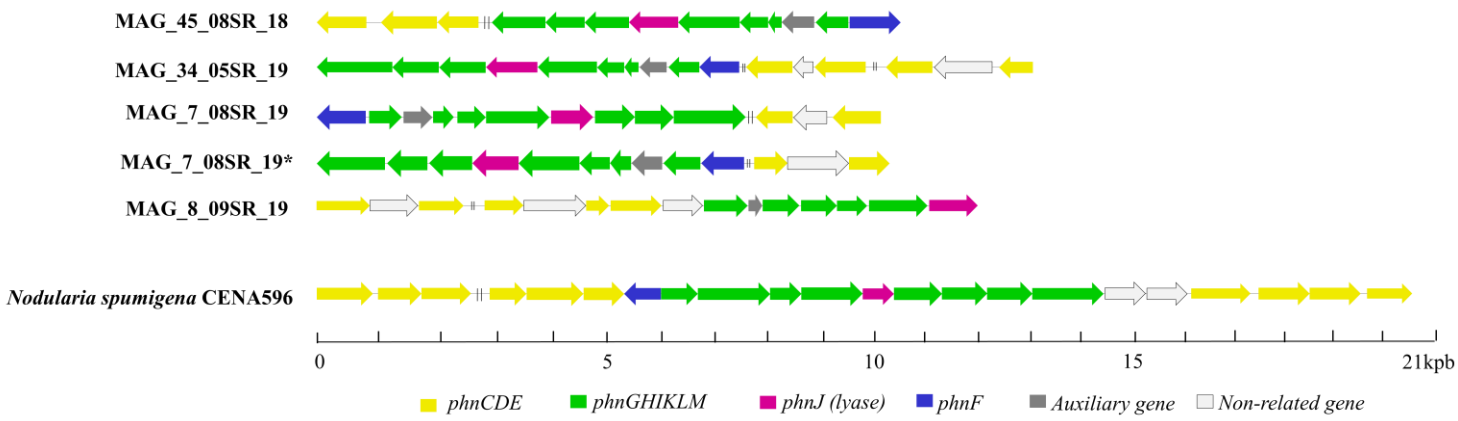

Figure 9. 'C-P lyase' cluster on cyanobacterial MAGs from Pantanal soda lakes. Nodularia spumigena CENA596 is used as a reference. (||) Genes found in different regions of the genome. (*) Duplicated cluster on the same MAG.

To refine the identification of the correspondent MAGs, we generated a phylogenetic tree based on *phn*J sequences, with a cyanobacteria database. Thus, we could analyze the relationship among lineages that carries the genomic ability to degrade phosphonates, and might likely be a non-classical methane contributor. The phylogenetic tree shows that MAG_45_08SR_18, MAG_34_05SR_19, MAG_7_08SR_19, and MAG_8_09SR_19, previously classified as Raphidiopsis genus grouped with the *Cylindrospermopsis* sp. lineages (Fig. 10). *Cylindrospermopsis* sp. is a very relevant group carrying the 'C-P lyase' enzymatic cluster among cyanobacteria as several lineages harbor it in their own genomes. We found two 'C-P lyase' clusters on MAG_7_08SR_19, with a different arrangement and, instead, is placed as a sister group with *Cylindrospermopsis* sp., *Raphidiopsis* sp., and *Nodularia* sp. lineages.

The cyanobacteria included in the tree with our soda lake MAGs have been previously described in literature to fix nitrogen or to have the genomic machinery to do so (Appendix F). *Nodularia spumigena* CENA596, as known here as our positive control was grouped with the same lineages. Teikari *et al.* (2018) performed *in vitro* assays, where they demonstrated *N. spumigena* UHCC0039 capacity of release methane as a sub-product of MPn degradation, which can confirm the credibility of our choice as control.

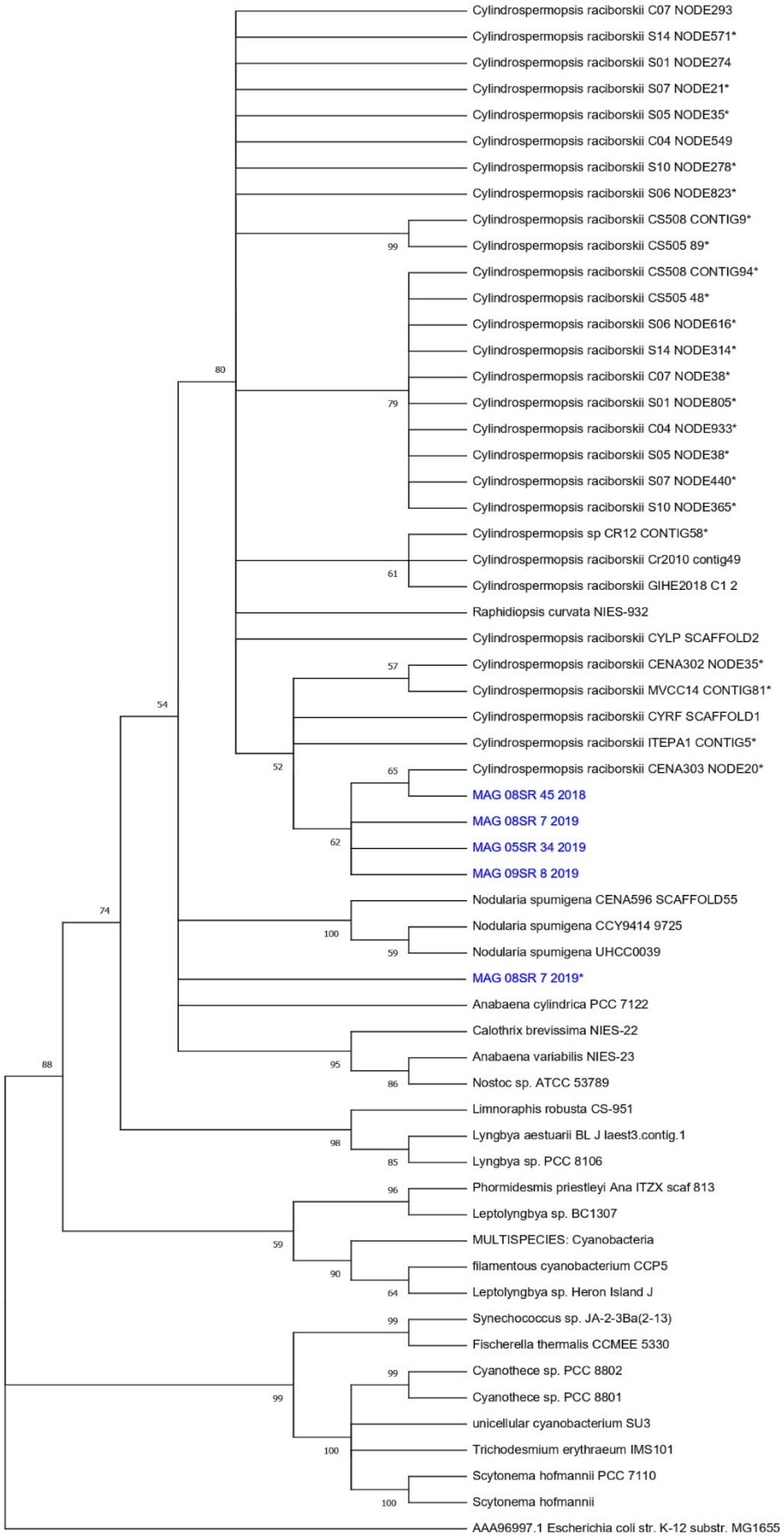

Figure 10. Phylogenetic tree for 'C-P lyase' gene marker *phn*J using the Maximum Likelihood method. Bootstrap inference values demonstrated at the branch nodes.

3.3.5. Chemical factors correlating to the potential of 'C-P lyase' pathway occurrence

The log-regression model aimed to evaluate the relationship between the relative abundance of *phn*J gene retrieved from metagenomic data, as previously presented, and organic phosphorous concentration (Fig. 11). The model indicates where a higher abundant 'C-P lyase community to increase the availability of orthophosphate ($P\text{-}PO_4^{-3}$), a preferable phosphorous source by microorganisms can be expected; this means that the genes would have a higher potential of being expressed where we find low concentrations of $P\text{-}PO_4^{-3}$ and thus P-limitation.

During the dry season (2017), we found a positive inclination (r^2=0.247, p=0.05), meaning that *phn*J abundance was higher when $P\text{-}PO_4^{-3}$ concentrations were not exactly limited. Contrarily, the flooded period shows a slim negative inclination among samples, with greatly lowered phosphorous concentrations and gene abundance. This drastic reduction of nutrient availability turns the year 2019 more opportune for our studied metabolism.

As we have an interest in cyanobacteria, we focus on some observations from the relationships presented in the eutrophic turbid lakes. Here, the model shows that lakes 08SR and 09SR are the ones with the highest potential for the 'C-P lyase' activation, as there is a quite low orthophosphate presence (Appendix B) and higher *phn*J gene abundance. Lake 04S, however, does not show lower concentrations of orthophosphate.

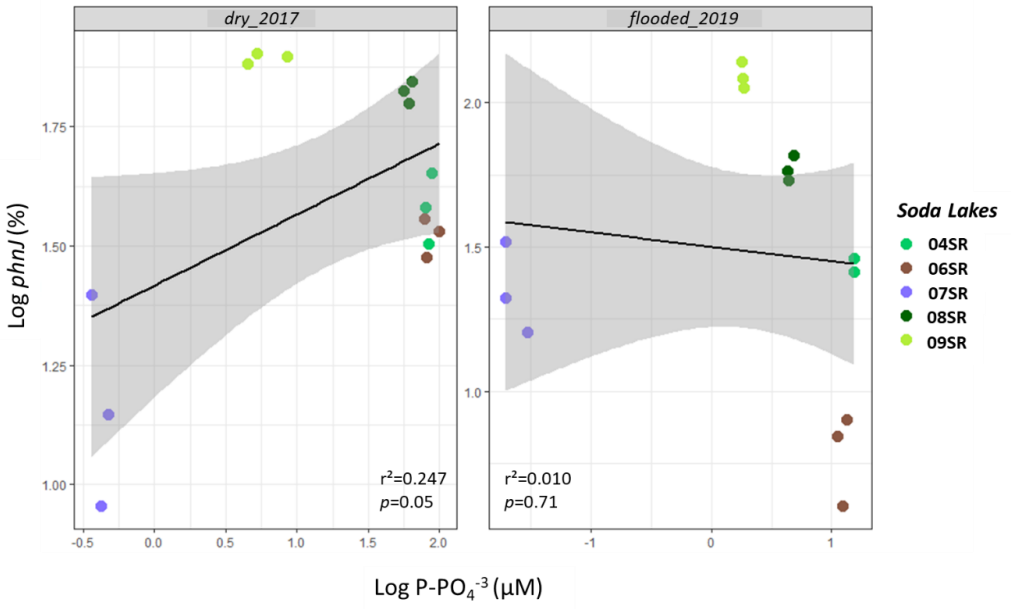

Figure11. Log-regression model for the relative abundance of the 'C-P lyase' *phn*J gene and ortho-phosphate ($P\text{-}PO_4^{-3}$) concentrations in 2017 and 2019. Data were transformed to $\log_{10}$.

Table 6 demonstrates the Pearson correlation for chemical parameters and relative abundance of the *phn*J gene to understand the strength of factors that could benefit or not the potential

for MPn degradation in the lakes. During both seasons, chlorophyll-*a* concentrations were not well related to gene abundance probably because the cyanobacteria that harbor the gene are not part of the bloom, so they do not impact the levels of the photosynthetic pigments. Furthermore, significant positive correlations to salinity and temperature and negative ones to iron concentrations were found.

During the drought period, nitrate and orthophosphate concentrations show a moderate positive and very weak negative relationship to *phn*J abundance, although not statistically significant. Because $P\text{-}PO_4^{-3}$ does not show a strong or significant correlation, we understand that this is indeed a factor that influences the 'C-P lyase' community, as shown above and in the literature. However, it is not so clear if it determines the potential of occurrence in the lakes during the drought period, especially an extreme one as it has been occurred in the Pantanal in 2017.

On the other hand, during the flooded season both nitrate and orthophosphate showed significant and negative moderate relationships to *phn*J gene abundance. This means when these nutrients are plenty, the 'C-P lyase' community is reduced. As we are dealing with metagenomic data, it does not mean that this pathway cannot occur, indeed the scarcity of ortho-phosphonate can promote the search for alternative sources of phosphorous. This correlation results in $P\text{-}PO_4^{-3}$ fits with the regression model previously presented.

Table 6. Pearson correlation with *phn*J relative abundance. Significance p > 0.05.

	2017 (drought)		**2019 (flooded)**	
	r	**p-value**	***r***	**p-value**
Chlorophyll-*a*	0,427	0,11	-0,202	0,46
Fe	-0,696	0,003*	-0,482	0,05*
NO^{-3}	0,497	0,06	-0,525	0,04*
$P\text{-}PO_4^{-3}$	-0,097	0,72	-0,502	0,05*
Salinity	0,716	0,002*	0,542	0,03*
Temperature	0,658	0,007*	0,507	0,05*

Fe: iron; NO^{-3}: nitrate; $P\text{-}PO_4^{-3}$: ortho-phosphate.

3.4. Discussion

Soda lakes present extreme physical and chemical conditions with peculiar biodiversity, representing very interesting environments to search for unconventional and biogeochemical processes. Phosphonate utilization is an alternative metabolic pathway for P acquisition in aquatic environments, and its degradation can result in releasing methane as a byproduct. To our knowledge, there is no information available in the literature on 'C-P lyase' activity in soda lakes. In this study, we explored the potential for methyl-phosphonate (MPn) demethylation, as an oxic methane production pathway in the Brazilian Pantanal soda lakes and focused on cyanobacteria's likely involvement in this process.

Metagenome shotgun sequencing allowed us to perform taxonomic and functional mapping of five soda lakes in two distinct seasons. Using a specific database reference for the *phn*J marker gene, we performed a screening to identify only the microbes capable of degrade MPn. The core bacterial community on the class level containing the *phn*J gene, here called the 'C-P lyase' community, consists of Actinobacteria, Alpha-, Beta-, Gammaproteobacteria, Cyanobacteria and Bacilli (Fig. 6). All of them are ordinary members of the bacterioplankton in soda lakes, known to be tolerant to high salinity levels (Andreote et al., 2018; Deshmukh et al., 2011).

The major cyanobacterial species known to perform OMP through the MPn demethylation pathway are *Nodularia spumigena, Trichodesmium erythraeum*, and *Synechococcus sp.* (Beversdorf et al., 2010; Gomez-Garcia et al., 2010; Teikari et al., 2018). A deeper analysis into the metagenomic data showed that cyanobacteria carrying the *phn*J in the Pantanal soda lakes belong to the order Nostocales, family Aphanizomenonaceae. Initially, we hypothesized that the hits corresponded to the bloom-forming species *Anabaenopsis* sp., particularly because they inhabit lakes 08SR and 09SR and form massive blooms in these lakes. However, after our efforts on screening several isolates, genomes and MAGs from the soda lakes, we found that these blooms don't have any potential to degrade phosphonates, at least not through the C-P lyase pathway.

Howbeit, taxonomic annotation identified five hits on four MAGs corresponding to *Raphidiopsis* sp., another member of the Aphanizomenonaceae family not yet isolated from the Pantanal soda lakes (Fig. 9). Unfortunately, we were not able to obtain the isolate for this strain. Recent literature has proposed merging the genus *Cylindrospermopsis* and *Raphidiopsis* based on phenotypical and molecular analysis showing that there are not many consistence differences among both – in this case, *Raphidiopsis* would be retained due to nomenclatural priority (Abreu et al., 2018; Aguilera et al., 2018).

Indeed, all sequences grouped within the clade of strains from the order Nostocales and four of them were closely related to *Cylindrospermopsis raciborskii* lineages (Fig. 6). As far as we known, the main studies on cyanobacterial isolates from the Pantanal soda lakes have never identified *Raphidiopsis* or *Cylindrospermopsis* members (Almeida et al., 2011; Andreote et al., 2018; Costa et al., 2016; Genuário et al., 2018b; Santos, 2013). Even though they are reported a freshwater strain, *C. racisborskii* is known for their physiological plasticity with plenty mechanisms of resistance that allowed them to spread into temperate regions (Bai et al., 2020).

Interestingly, four strains grouped within *C. raciborskii* mainly isolated from South America and were closely related to strain CENA303 (Fig. 10). CENA303 is a South American non-toxic strain, previously isolated by our working group from a freshwater reservoir at Rio Grande do Sul, Brazil (Abreu et al., 2018). In culture, CENA303 performed distinctly, as it don't show heterocyst formation, hence is not able to fix nitrogen. Abreu *et al.* (2018) points out that the South American *C. raciborskii* strains show a divergent genome compared to those isolated from other areas, like Australia and Asia. In our study, we could identify the *phn*J gene from these cyanobacteria in the

Brazilian Pantanal soda lakes and show that they are closely related to other South American strains well, representing the expected biogeographical pattern.

With this biogeographical driver of *C. racisborskii* genomes, some strains can adapt very well in water systems where phosphorous concentrations highly fluctuates (Piccini et al., 2011), and this might favor their presence on the soda lakes. One relevant feature of this cyanobacterium is their high phosphate uptake and phosphate storage capacity (Amaral et al., 2014; Willis et al., 2018). Although Pi is the preferable source of phosphorous for them, a few studies have shown that *Cylindrospermopsis* genera can efficiently consume dissolved organic phosphorous, including phosphonates compounds, such as glyphosate and 2-aminoethyl phosphonate, when under Pi scarcity (Bai et al., 2014; Miotto et al., 2017). Willis et al. (2018) reported the presence of the complete cluster for the 'C-P lyase' pathway to consume phosphonate in the genomes of Australian strains of *C. raciborskii*. However, we did not find any information about their capacity to degrade MPn and subsequently release methane. This will be one of our next efforts to contribute more information on this topic, considering that *Cylindrospermopsis* sp. not only might form massive blooms, but also produce toxins that are a healthy risk to human and wildlife.

In none of the other lakes we found cues of cyanobacteria containing the *phn*J gene, at least in none of the periods we have evaluated. This is interesting because it demonstrates that the lakes represent much more complex environments in terms of physical and chemical characteristics, reflecting on functional traits and microbial composition. In fact, lake 04SR is also harbors cyanobacterial blooms and no hits of Cyanobacteria nor MAGs related to *Raphidiopsis* sp. were found in there.

As cyanobacteria are responsible for the blooms are not part of the 'C-P lyase' community, the association with heterotrophic bacteria can be indicative for this pathway. Heterotrophic bacteria associated to Cyanobacteria are responsible for the degradation of a major proportion of the particulate organic matter in lacustrine ecosystems (Parveen et al., 2011). For example, the bacterioplankton interacting with *Trichodesmium* sp. on Pi-depletion conditions have been identified as a source of methane in waters richer in MPn than Pi (Carini et al., 2014). Other studies performing *in vitro* assays indicate that when in consortium with planktonic cyanobacteria, heterotrophic bacteria are the main strains in marine environments to utilize MPn and release methane, likewise (Khatun et al., 2019; Repeta et al., 2016). This is why we need to access the whole community performing this alternative metabolism, especially when examining the different soda lakes.

In fact, the 'C-P lyase' community we found in the soda lakes is frequently described in aquatic environments to actively contribute to methane supersaturation in oceans and temperate lakes (Chin et al., 2016; Karl et al., 2008; Villarreal-chiu et al., 2012; Y. Wang et al., 2017). Marine environments have more available data regarding the usage of the *phn*J gene and OMP, where the taxa are frequently attributed to Proteobacteria, Actinobacteria, Bacilli, Chloroflexi, Clostridia and Cyanobacteria (Villarreal-chiu et al., 2012). Freshwater environments, such as Lake Matano

(Indonesia) and Lake Greifensee (Switzerland), described a similar composition as well (Huntscha et al., 2018; Yao et al., 2016); nonetheless there is no information regarding soda lakes. Obviously, we detected an ordinary microbial community for OMP through phosphonate demethylation, and their specific activity in each environment is controlled by the physical, chemical and biological characteristics.

Looking close to the 'C-P lyase' community, during the drought season, two classes demonstrated a considerable higher abundance in all the lakes, i.e. Gammaproteobacteria and Actinobacteria (Fig. 6). Gammaproteobacteria is major group with representatives on MPn demethylation in many aquatic systems (Dyhrman et al., 2009; Yao et al., 2016). They are often alkaliphilic and halotolerant bacteria, highly abundant in soda lakes and other hypersaline environments, and known to greatly participate in several metabolic pathways (C, N, and S) as chemoorganotrophy (Liu et al., 2018; Sorokin et al., 2014b). In fact, in Lake Bonney, a cold hypersaline freshwater system in Antarctica, the main isolates capable of contributing for this OMP process are from the phylum Gammaproteobacteria (Li et al., 2020).

Similarly, Actinobacteria are cosmopolitan residents in aquatic ecosystems, well adapted to limiting nutritional conditions, and fluctuations on pH, temperature, and salinity (Allagier and Grossart, 2006; Ávila et al., 2017; Jiang et al., 2010). These traits might have favored Actinobacteria's dominance during the dry season. They are capable to degrade several complex organic compounds, such as the agrochemical, glyphosate (Barka et al., 2016; Jiang et al., 2010). Additionally, Actinobacteria seem to not only consume phosphonates in the soda lakes, but may also produce it. Yu et al. (2013) detected the production of active form of phosphonates in many Actinobacteria isolates, regarding the presence of the gene *pep*M (phosphoenolpyruvate mutase). This group is commonly reported as major natural producer of a variety of phosphonates, *in vitro* and *in situ* conditions (Gao et al., 2014).

As the season changed, the environmental conditions in the soda lakes showed an increase in pluviosity and water levels, leading to a reduction in nutrients (Appendix A). During 2019, the microbial dominance changed altogether in all the soda lakes, when Bacilli showed a considerable increase in abundance, dominating the 'C-P lyase' community. In soda lakes, including the ones from Nhecolândia, Bacilli are commonly related to nitrogen fixation (Sorokin et al., 2014b). They are alkaliphilic organisms, capable of producing stable enzymes, such as hydrolases, at high pH levels (Martins et al., 2001).

Once again, the soda lakes shared a 'C-P lyase' community, thus the dominant organisms on this role change from dry to flood seasons. Navarro et al. (2009) showed that in saline systems, hydrological cycles are a greater modulator of microbial communities, restricting their activities during drier periods to those which can adapt to conditions of scarcity. Microbes can change their nutritional requirements or even, expand the type of substrates they can use as sources for growth. The organisms capable to use phosphorous in its diverse forms, including phosphonates, show a high

plasticity and convey an ecological advantage over the ones who cannot. Even though none of the soda lakes can be characterized as oligotrophic environments, there is a great fluctuation of nutrients (including organic P), that may favor certain adaptative strategies and/or opportunistic microorganisms prepared for scarcity conditions (Livermore et al., 2014).

Functional redundancy refers to the ability of a community to maintain a functional stability despite loss or change in community members, which enable resistance and resilience to many disturbances (Ren et al., 2019). Saline environments are very predisposed to suffer fluctuations in their physical and chemical parameters between dry and rainfall periods, which also modulate microbial community structures and composition (Schagerl et al., 2015). In this study, variable pluviosity in the region changed nutrient and salinity levels of the soda lakes differently and thus can be regarded as a "disturbance" acting on the selection of specific functional groups of microbes.

Hydrological characteristics of the Pantanal wetlands might also affect microbial community composition in the lakes, where diversity is commonly lower during drought periods as the extreme conditions increase (Malone et al., 2012b). Contrary, alpha-diversity and gene abundance were higher during drought in our case (Fig. 7), but it is important to remember that we are evaluating a specific community and metabolism, not the entire microbial composition. Also, as an alternative pathway for phosphorous, it is quite comprehensive that during an inhospitable condition, remaining opportunistic competitors have access to surviving traits for plenty adverse situations.

During periods of elevated pluviosity levels, phytoplanktonic blooms can terminate due to dissociation of cells, leading to alterations of bacterial community composition (Schagerl et al., 2015). Moreover, at habitats with high primary productivity, such as the soda lakes, allochthonous organic matter and other released metabolic by-products can normally impact bacterioplankton abundance and composition, which can also affect selection of functional groups (Dimitriu et al., 2008; Niu et al., 2011). This could also explain why the alpha-diversity on lakes with strong blooms was found to be greater concerning this pathway, in comparison to mild- or no-bloom presence (Fig. 7).

Dissimilarity on beta diversity was drawn across seasons on the soda lakes, although changes in composition were very similar at the class level for all five studied lakes (Fig. 8). Thus, albeit, their exclusive physical and chemical characteristics, extrinsic factors and trophic interactions in each lake might vary simultaneously, leading to a spatial-temporal synchrony over community composition (Kent et al., 2007). Among the gradients at our soda lakes, we determined nutrient availability; organic matter quantity and quality; zooplanktonic and viruses grazing; water temperature; hydrological fluxes; succession; salinity changes; oxygen levels and stratification (Adams et al., 2010, 2015; Crump et al., 2003; Mueller-Spitz et al., 2009; Sorokin et al., 2014b; Jingxu Zhang et al., 2014)

After bring up the likely interference of the soda lakes characteristics on the microbial community structure and composition, we need to figure the actual potential for the usage of the 'C-P lyase' pathway. As this OMP pathyway is strictly correlated to phosphorous scarcity, its availability is important for the discussion of what is affecting its potential of occurrence in the soda lakes. The main

preferable form of phosphorous used by microbes is inorganic phosphorous (Pi), as it is readably for assimilation, being a major limiting nutrient in aquatic ecosystems (Kuhn et al., 2019). When integrated into biomass, the phosphorous will be released to the environment in several organic forms, after cell death and exudation (Kuhn et al., 2019).

Phosphonates are likely to be an intracellular storage for P incorporated on cellular structures and due to its complex composition, it is not easy to degrade. Dissolved phosphonate molecules are suspected to comprise novel polysaccharides, which represents a large fraction of semi-labile dissolved organic matter (DOM) in marine environments (Repeta et al., 2016). Specifically, MPn must be dissolved in water for being consumed, mostly after cell death, releasing methane before it reaches the dissolved form (Del Valle & Karl, 2014). For shallow lakes, though, there may be an endogenous circulation mechanism of phosphorous due to their short stratification. Jin et al. (2019) considerer that water level fluctuations, which can promote material circulation in the lake, concentrating or diluting the nutrients available for the microbes, and influencing bacterioplankton composition and succession as well. Moreover, for the bloom-presenting lakes, these water fluctuations and abrupt water chemistry changes could lead to release of intracellular compounds, such as phosphonates, in the chemocline region of the soda lakes (Fazi et al., 2021).

Furthermore, alkalinity can be a point in favor for MPn usage in the soda lakes. Although organic phosphorous is relatively stable in a water environment and cannot be directly absorbed by phytoplankton, they are easier to mineralize and decompose under alkaline and high temperature conditions (Kong et al., 2018). Degradation of MPn is faster under alkaline conditions than under acidic conditions (Xia et al., 2019). This feature would reduce the potential for *phn*J gene expression in lake 07SR, as it is much less alkaline.

However, the usage of phosphonates has been only studied in deeper water systems than our soda lakes. Methane accumulation in oxic layers has been reported in a depth around 7-9 m in freshwater systems and 200-300 m in seawaters (Grossart et al., 2011; Tang et al., 2016). Sosa *et al.* (2017) data strongly indicates the Phn cycling mainly occurs at least 200-300 m, being an important source of MPn in the deep ocean as well. The fact that the soda lakes are shallow (up to 3 m) with a slight stratification process makes it difficult to determinate which layer would be appropriate for oxic methane production from methylphosphonate degradation. What we consider here is that the nutritional composition of the lakes would allow the potential 'C-P lyase' community to act when necessary.

Based on that we performed a logarithmic regression to understand which lakes would have a higher potential for the *phn*J gene to be active and consuming phosphonates. Literature data leads as to expect that the lakes with lower or relatively lower orthophosphate concentrations and higher *phn*J abundance might have a likely activity of alternative pathways for phosphorous; e.g. the 'C-P lyase' demethylation pathway. Based on our current understanding, both regression models show that lake 09SR might be the one with the highest potential for the 'C-P lyase' pathway as the phosphorous

supply mechanism, and a possibility acting as OMP pathway as well (Fig. 11). In addition, the negative correlation between *phn*J presence and ortho-phosphonate concentration during 2019 (Table 6) is coherent as well for lake 09SR potential, corroborating the concept that alternative sources of organic phosphorous are needed when there is a scarcity of readily usable phosphorous for the microbial community. Therefore, as we see lower concentrations of nutrients during this partly flooded season, the conditions were favoring an increase in the abundance of microorganisms able to use substitute sources of phosphorous, such as phosphonates. Sosa *et al.* (2020) brought more evidence that a negative correlation between *phn*J gene and Pi concentrations are favoring phosphonate degradation, and contribution to methane productions.

The negative correlation with nitrate in the same period of 2019 also reinforces the conditions of such environments. Additional carbon and nitrogen can be drivers for MPn consumption, reducing the available Pi, and stimulating the growth of microbes which can be used as alternative sources of phosphorous, followed by methane release (Martínez et al., 2013; Ye et al., 2020). The colimitation of N and P during stratified bloom periods might accelerate the methane release using MPn, where excess nitrogen promoted P depletion, influencing the microbial search of alternative P-sources (Khatun et al., 2019). From that we can understand that in lakes with massive cyanobacterial blooms, such as 08SR and 09SR, an additional source from C-P lyase activity is available.

This could also mean a likely indirect influence of the *Arthrospira sp*. and *Anabaenopsis* sp. blooms to OMP through methylphosphonate demethylation. When nitrogen fixation is happening, other bacterial groups may be benefit of N entrance (Del Valle & Karl, 2014). Thus, complementary to nutritional conditions, the ability of *Anabaenopsis* sp. to fix nitrogen on the soda lakes could induce other bacteria to use phosphonate as P source (and possibly, release methane as well). In addition, H_2 byproduct from N-fixation has also been reported to be used by archaea as a source for methanogesis (Table 2), enhancing the associative methane production.

Together with N, iron (Fe) concentrations can also affect MPn consumers potential, as this ion catalyzes the C-P lyase. Thus, it induces the 'C-P lyase' pathway when activating the enzyme on its 4Fe-4S activation center (Kamat & Raushel, 2015). Indeed, in cultures of *T. erythraeum*, *phn*D and *phn*J were not expressed under P-depleted and Fe-deficient conditions (Dyhrman et al., 2006). In fact, Fe is also a catalyzer for nitrogenase activity, stimulating N_2 fixation by diazotrophic organisms (Li et al., 2020). This can increase the requirement for Pi as the environment turn into a limiting source. Sosa 2019 brings up that if the correlation of Fe and *phn*J gene abundance is negative, as we also identified for our data (Table 6), this nutrient is highly to be catalyzing the C-P lyase pathway.

We are very aware that our inferences could be stronger if methane data was fully available. During 2017, there wasn't enough depth to sample methane values, so it was not possible to carry it out. For 2019, we showed on the previous chapter that methane was indeed higher from bloom-presenting lakes. Even though we do not have the concentrations of lake 09SR, due to sampling issues, we were still able to correlated the 'C-P lyase' pathway with the presence of blooms on our general

analysis (Fig. 4). Therefore, from this result, we believed it was pertinent to carry on the investigation on MPn demethylation focusing on the drivers for it to happen than just relying on methane data.

Another issue to be taken in our study about quantifying methylphosphonates dissolved in the soda lakes. The most efficient methodology used to determinate phosphonates in general is nuclear magnetic resonance (Forlani et al., 2011a; Ilikchyan et al., 2009; Repeta et al., 2016); unfortunately, we were not able to perform this analysis due to the lack of available equipment. Yet, we also believe that as C-P lyase is part of phosphorous cycling, our connections to this nutrient availability are also powerful on comprehending its potential. Additionally, to contributing to OMP, another possibility is that microbes use phosphonate not exclusively to obtain P, but also to consume carbon and nitrogen, and achieve the energy stored in the complex bond among C-P that these compounds present (Sosa et al., 2017). In this case, MPn degradation might not lead to methane productions, having an impact on phosphorous cycling but not much on greenhouse gas contributions – something else included in our future investigations.

3.5. Conclusions

In this study we concluded that:

- In the soda lakes, there is a microbial community able to perform the 'C-P lyase' pathways, that changes with different seasonal conditions, but remains more abundant in the presence of cyanobacterial blooms;
- Although the cyanobacterial blooms are not part of the 'C-P lyase' community, our metagenomics and MAGs analyses show that *Raphidiopsis* sp. is representing these taxa, and their future experimental investigations are worth to be done;
- According to the data regarding phosphorous metabolism, the partly flooded season and lake 09SR presents the nutritional and chemical conditions for the 'C-P lyase' pathway to be required;
- Even with lacking specific analysis, we believe that this OMP can be occurring on the soda lakes, however, with not a significant direct participation of cyanobacteria.

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4. CHAPTER 3: SHORT-TIME MONITORING OF OXIC METHANE PRODUCTION OF CYANOBACTERIA ISOLATES FROM BRAZILIAN SODA LAKES

Abstract

Oxic methane production has been reported as a great contributor to methane emissions from oxic surface waters. While some mechanisms of oxic methane production related to demethylation have been recently resolved, photosynthesis-associated methane production remains enigmatic. In this study, we used three bloom-forming, two unicellular, and one filamentous cyanobacteria from the Pantanal lakes to assess their ability to produce methane under in vitro conditions. The gas concentrations were measured in a dark/light photoperiod, using membrane-inlet mass spectrometry. All strains produced methane during the photoperiod, with a rapid decrease in rates with the onset of dark. Methane production rate by the two filamentous Cyanobacteria was significantly higher than that of the unicellular ones. Long-term experiments in which the photoperiodicity was inverted revealed that methane production continues for ca. 24 h following the previous light regime, till it adjusts to the new one. This suggests a link between methane production and the cyanobacteria circadian clock. Furthermore, this indicates that photosynthesis-associated methane production may be linked to the dark to many other mechanisms than just photosynthesis. While not resolving the mechanism of photosynthesis-associated methane production, our results support the notion that cyanobacteria produce substantial amounts of methane under oxic conditions, in a light-fueled mechanism.

Keywords: Photoperiod; Circadian clock; Reactive oxygen species; Non-enzymatic; Savitzky-Golay filter

4.1. Introduction

Cyanobacterial contribution to methane emissions has been recently suggested to occur in many environmental conditions where classical pathways of methanogenesis would hardly happen due to the high concentration of oxygen in the surrounding water. Using membrane-inlet mass spectrometer (MIMS), Bižić *et al.* (2020) demonstrated methane production associated with light exposition during primary activity by cyanobacteria from a plethora of environments. Their main findings suggested that metabolic products generated during photosynthetic carbon fixation might be used as precursors for methane formation by yet unknown mechanisms.

The MIMS apparatus allows the continuous online measurements of methane and oxygen concentrations from cyanobacterial cultures through an extended time period. This technique's main advantage is the constant in situ measurement of dissolved gases in a microbial suspension, allowing to monitor several biological gases simultaneously with a high temporal resolution of a few seconds (Burlacot et al., 2020; Dmitriy Shevela et al., 2018). MIMS is being applied to studies regarding gases produced during photosynthesis, enzymatic activity, inorganic carbon transport, hydrogen production, and artificial catalysts for water oxidation (Beckmann et al., 2009; Burlacot et al., 2020).

Following the inferences made in Bižić *et al.* (2020) together with the previous studies relating methane emissions from soda lakes and cyanobacterial blooms, we proposed to investigate isolates from the Pantanal soda lakes for their potential of CH_4 production in oxic waters (OMP). Considering the high primary production levels of cyanobacteria in the soda lakes, we hypothesize that cyanobacteria strains isolated from the lakes can produce methane concomitant with signals of photosynthesis, substantially contributing to the methane ratesdescribed for the Pantanal wetlands.

4.2. Methods

4.2.1. Cyanobacterial strains

The cyanobacterial strains were isolated from soda lakes located in Pantanal's Nhecolândia subregion (Aquidauna, Mato Grosso do Sul, Brazil). Table 7 presents a brief description of the chosen cyanobacteria for this study, which are also represented in Figure 12. The strains were cultivated at 22 ± 1°C, under light radiation of 40 µmol photons.m^{-2} s^{-1} and a photoperiod regime of 14:10 h light:dark in Z8 growth media. All cultures are from the 'Cyanobacterial Culture Collection' at CENA/USP, under the supervision of Prof. Dr. Marli F. Fiore.

Table 7. Cyanobacterial strains isolated from the Brazilian Pantanal's Nhecolândia subregion.

Cyanobacteria	Strain ID	Location	Description
Anabaenopsis elenkinii	CCIBt3563	Salina do Meio	Bloom-forming nitrogen-fixing planktonic
	CENA651	04SR, Lagoa Alongada	
Arthrospira platensis	CENA650	08SR, Lagoa Lobo-Porco	Bloom-forming non-nitrogen-fixing planktonic
Geminocystis sp.	CENA526	Salina Centenário	Unicellular non-nitrogen fixing non-bloom forming
	CENA649	Not found	
Phormidium sp.	CENA622	Lakeshore, Salina Grande	Non-nitrogen-fixing filamentous strain

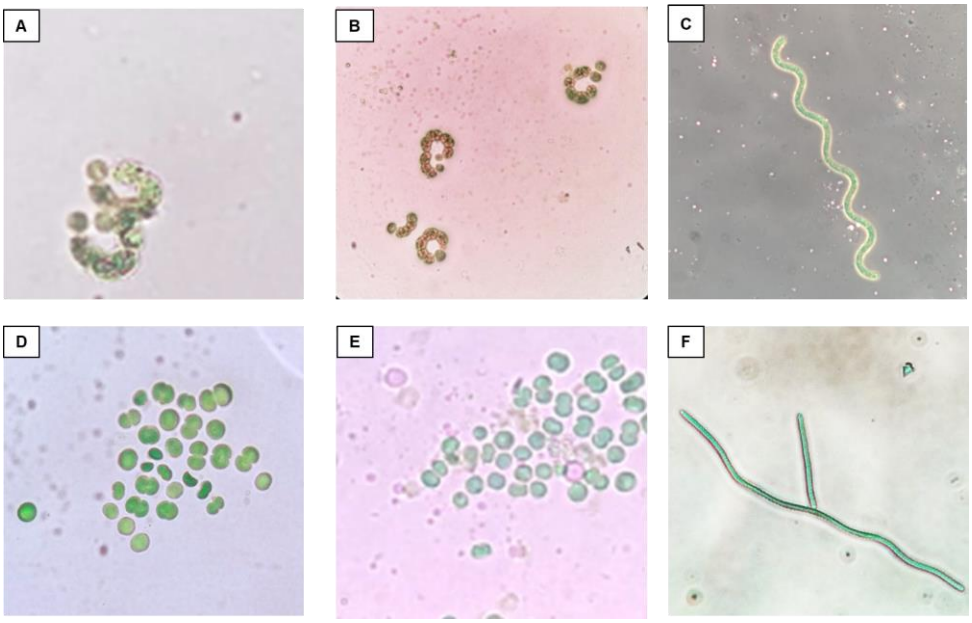

Figure 12. Optical microscopic images of the cyanobacterial strains described on Table 7. A. *Anabaenopsis elenkinii* CCIBt3563 (100x); B. *Anabaenopsis elenkinii* CENA651 (100x); C. *Arthrospira platensis* CENA650 (40x); D. *Gemonocystis sp.* CENA526 (100x); E. *Geminoscytis sp.* CENA649 (100x); F. *Phormidium sp.* CENA622 (40x).

4.2.2. Strains cultivation and purification

Purification procedures were carried out after the transport of the samples to the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB, Stechlin, Germany). Two approaches were used to recover the cultures. A washing procedure was conducted at least twice as described in Heck *et al.* (2016), with a centrifugation process switched to vacuum filtration or serial dilutions from 1:10 to $1:10^{-6}$ mL as an alternative. Due to issues with recovery time, it was not possible to make the cultures axenic, but contamination was reduced to a large extent by constant dilution.

At IGB-Stechlin, the cultures were incubated at 18 ºC, under light radiation of 40 µmol photons m^{-2} s^{-1} and a photoperiod regime of 14:10 h light:dark in Z8 media culture. After purification procedures, the cultures were monthly maintained for the following experiment.

4.2.3. Identification of methanogenic and methanotrophic genes

As the cultures were not axenic, molecular screening for target genes was performed to identify the presence of methanogenic and methanotrophic organisms. The DNA was extracted using the phenol-chloroform protocol modified from (Nercessian et al., 2005), and quantified by fluorometry with QuantiFluor® dsDNA kit (Promega, USA) using Quantus Fluorometer (Promega, USA). Afterward, amplicon PCR for methanotrophy (*pmo*A) and methanogenesis (*mcr*A) genes was performed. For *pmo*A, the primers set used were A189-f (GGNGACTGGGACTTCTGG) and MB661-

r (CCGGMGCAACGTCYTTACC), with the following reaction conditions: 95 °C for 4 min; 40 cycles of 95 °C for 30 s, 58 °C for 45 s, 72 °C for 45 s, and a final elongation step at 72 °C for 10 min (Costello & Lidstrom, 1999; Holmes et al., 1995). For *mcr*A reaction, the primers sets mlas-f (GGYGGTGTMGGDTTCACMCARTA) and mcrA-r (CGTTCATBGCGTAGTTVGGRTAGTT) were used, with the following adapted reaction conditions: 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and a final elongation step at 72 °C for 10 min (Angel et al., 2012; Steinberg & Regan, 2009).

4.2.4. Methane continuous quantification by membrane inlet mass spectrometry (MIMS)

Gas methane quantification was performed using a MIMS instrument, a mass spectrometer QMG 220 M1, PrismaPlus®, C-SEM, 1-100 amu (Pfeiffer Vacuum, Germany) connected to a Pfeiffer Vacuum HiCube 80 Eco turbopump (Fig. 13). For the methane measurements, the cultures were prepared as follows. About 15 mL of cyanobacterial culture were washed 3-4 times with autoclaved and filtered Z8 media to remove any methane previously present. Afterward, the cells were concentrated into 5.5 mL for the experiment, from which 3.5 mL were transferred to the culture chamber on the MIMS setting, 1 mL was used for chlorophyll *a* measurement, and 1 mL for DNA extractions. The culture chamber was placed on a magnetic stirrer for constant mixing to avoid sedimentation of cells, and its temperature was maintained at a range of 26-29 °C by a water bath externally connected.

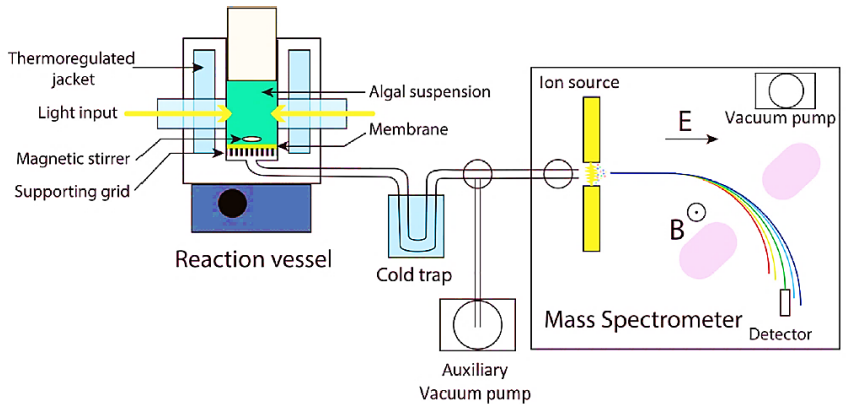

Figure 13. A membrane inlet mass spectrometry (MIMS) was set up and retrieved from Burlacot *et al.* (2020).

Two experiments were executed under the same light regime as for cultivation, i.e. 14:10 light:dark photoperiod. Experiment 1 aimed to quantify methane production and compare different strains of cyanobacteria, including strains from the same genera, for 48-hours of observation. Here, we

used as controls, an analysis at a 48-h light and another at a 48-h dark period. MiliQ water was also run as a standard control for checking the precision of the measurements.

Experiment 2 was conducted after identifying methane production. A bloom-forming strain *Arthrospira platensis* CENA650, a filamentous strain from the lakeshore *Phormidium* sp. CENA622, and a unicellular benthic strain *Geminocystis sp.* CENA526 was tested on the MIMS for five, eight, and four days, respectively. With these longer reruns, we aimed to retrieve more detailed information on the production rate of methane that was observed in Experiment 1. For both experiments, we started at the dark phase to prevent immediate stress on the strains.

4.2.5. Chlorophyll-*a* measurements

To be able to compare strains' activities, chlorophyll-*a* levels were measured. The samples were filtered onto GF/F filters (Millipore), to which 1.5 mL of 90% acetone was added. All samples were vortexed for 30s and stored at 4° C for 24-h in the dark, as described in Shrestha (2018). After 24-h, the samples were centrifuged at 3,000 rpm for 30 minutes. On the spectrophotometer Hitachi 2900, the wavelengths measured were 630, 645, 665, and 750 nm. Procedures and calculations were done following Strickland & Parsons (1972) with minor modifications for the volumes used.

$$Chla\ mg/m\text{^}3 = \frac{Ca * v * 1000}{l * V}$$

$$Ca = 11.6 * (E665 - E750) - 1.31 * (E645 - E750) - 0.14 * (E630 - E750)$$

E = wavelength
v = volume acetone extract (cm^3);
l = cuvette (cm)
V = volume of sample (cm^3)

This procedure was performed before and after incubation on the MIMS to identify changes in cyanobacterial photosynthetic activity through the experiment. In order to obtain the variation through time of chlorophyll, we used a linear extrapolation from the start to the end, and from this function it was extracted the amount of chlorophyll for each point in the measurements.

4.2.6. Methane production rates calculations

Methane and oxygen concentrations were calculated, based on known solubility values and standardized by concentrations measured in the MiliQ water control at the same temperature range and salinity levels on the media. Methane production rate per hour per µg of chlorophyll was calculated using the 1st derivative of the Savizky-Golay polynomial filter in the signal() (*https://cran.r-project.org/web/packages/signal/index.html*) package in R 4.0.5 (R Core Team, 2020). Over the filtered data, it was used LOESS (locally weighted smoothing) smoothing tool for a non-parametric regression data. Visual graphics were generated in the software SigmaPlot 14.5 (Systa Software, San Jose, CA).

4.3. Results

4.3.1. Search for methanotrophs and methanogens

Even though we invested quite some effort to purify all cultures, it was not possible to make them axenic in the time frame scheduled for the experiment. For this reason, we investigated the presence of methanotrophic and methanogenic organisms by PCR amplification of the target gene *pmo*A and *mcr*A, respectively.

The screening results were evaluated considering the amplification of fragments of the same size as the primers used for each amplicon investigated. For *pmo*A, amplification was positive for 472base pairs, as indicated by the green arrow in Figure 14, and observable on the positive control sample. Here, we found methanotrophy in *Anabaenopsis elenkinii* CENA651 and CCIBt3563, and *Phormidium* sp. CENA622, during all conditions tested. Also, *Geminocystis* sp. CENA649 at 24h dark control condition revealed methanotrophy.

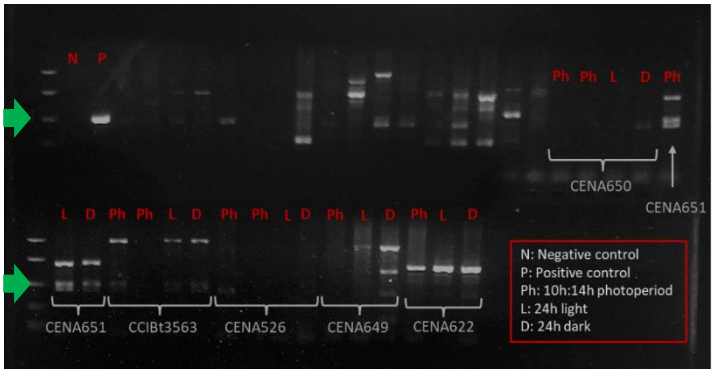

Figure 14. Electrophoresis gel for PCR analysis for the marker gene *pmo*A for methanotrophic organisms.

To verify whether archaeal organisms were also present in the cultures, we screened for the marker gene *mcr*A of 469 base pairs, as indicated by the green arrow, according to the size of the primers used and the positive control result (Fig. 15). None of the cultures used for the incubation assay tested positive for the archaeal methanogenic searching. This result is important for our inferences on methane production being exclusively coming from cyanobacteria activities.

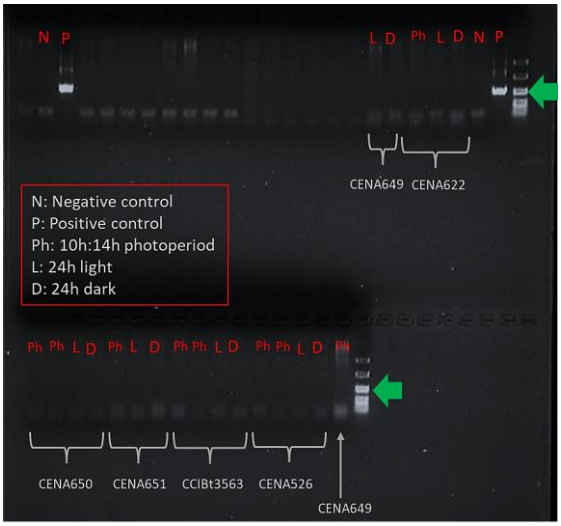

Figure 15. Electrophoresis gel for PCR analysis for the marker gene *mcr*A for methanogenic archaea organisms.

4.3.2. Experiment 1: Comparing distinct cyanobacterial cells for methane production

Before analyzing our results, it is important to clarify the methane production rate curves we obtained. They resulted from a mathematical expression of the hourly change of methane concentrations per gram of chlorophyll. Therefore, for the methane concentration slope, when a peak of gas occurred, it should be read as an absence of production, which means that the rate should be equal to zero. Another characteristic of the slopes for methane rates is the fluctuation of the gas for all samples, which can be happening due to oxidation processes or degassing from the system changing the gas saturation inside the incubation chamber.

At first, we will focus on Figure 16, which shows oxygen concentration, methane concentration, and production over 48 hours, comprising a photoperiod of 10h:14h dark:light. For all strains, we can see that during the light phase, there is an increase in oxygen concentrations (blue) which starts to decline when the lights are turned off. This effect can be related to the photosynthetic activity, as it mostly happens under light availability to be used as an energy source by the cyanobacterial cells. The methane concentration slopes (purple) alone are generally not well defined with the dark:light phases, especially for the filamentous strains. For CENA526 and CENA649, it is observable that methane concentrations rise in the presence of light, and decrease when they are turned off. All filamentous strains tested showed higher rates of methane production when compared to the unicellular ones.

For both *A. elenkinii* strains, as well as CENA526, we observed that in the second light phase, a drastic drop in the concentrations of oxygen occurred. *A. elenkinii* CENA651 and CCIBt3563 seemed to be very sensitive to the light intensity used in the experiment, so they might not have been

able to perform photosynthesis properly, especially during the second light event. Even though, the peaks in methane production occurred during the light period, they followed the oxygen peak.

A. platensis CENA650, we observed several peaks in methane production during the dark period as well, when oxygen concentrations approached zero. This strain is the one repeatedly showing peaks of production, appearing to be likely active in generating methane, when compared to the others. *A. platensis* is a non-toxic filamentous cyanobacterium responsible for the major blooms in the Pantanal soda lakes, but not able to fix nitrogen (Santos, 2013). Also, the highest methane values and rate is from this strain.

Phormidium sp. CENA622 is another filamentous strain, however, it was isolated from the lakeshore, not composing the cyanobacterial blooms at the soda lakes. Here, the peaks of methane production are mostly restrained to the light phase and in the presence of oxygen. There is a small delay between the light periods, which can be due to some difficulties in strain acclimation to the experimental conditions.

Besides filamentous and bloom-forming cyanobacteria, we also tested two unicellular *Geminocystis* sp. strains commonly present in the soda lakes. For both strains, the methane production rate was the lowest compared to the other tested strains after the normalization of the measured values based on chlorophyll-*a* values, reproducing the relevant variation in cell morphology and hence biomass. Most of the methane production peaks occurred in the presence of light leading to higher concentrations of methane. Although, interestingly, both show a peak right at the end of both dark phases.

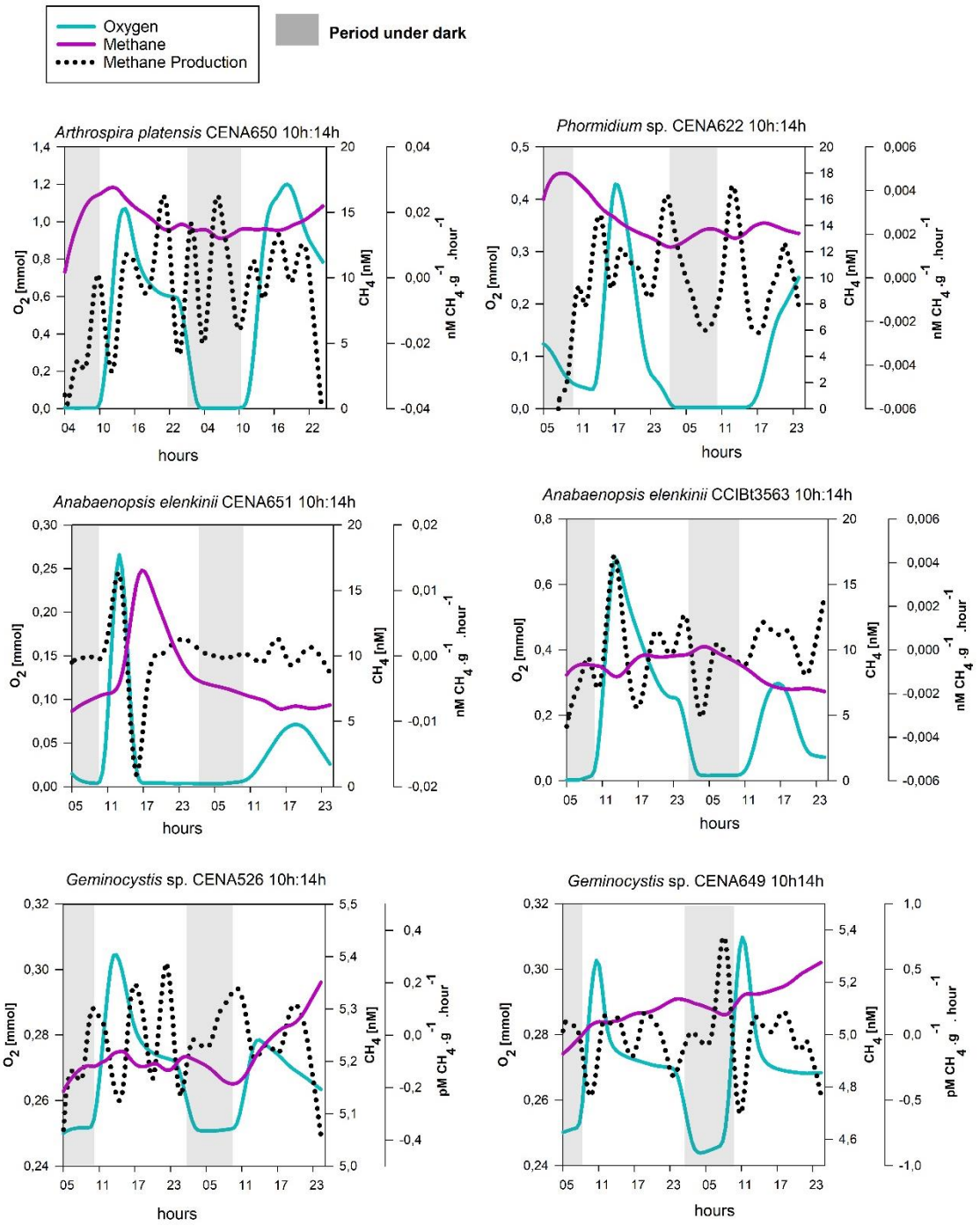

Figure 16. Continuous measurements of oxygen concentrations (blue), methane concentration (purple), and production rate (dotted black) under 10:14 dark:light photoperiod using a mass-inlet membrane spectrometry (MIMS). Light gray background colors refer to the dark phase.

Because the photoperiod results showed that methane production was mostly present when the lights were on and in the presence of oxygen, it was expected that in the 24-h light controls, the methane rate would be constant. However, we found a great fluctuation of the slopes for oxygen and methane concentrations for all strains (Fig. 14). Strain CENA649 unexpectedly seems to act following the light and dark alternation phases for oxygen production, as it shows peaks and trough following what it appears to be the photoperiod they were incubated. Similarly, CENA650 and CENA526

demonstrated an initial increase in oxygen levels, but both starts to decrease until 24-h later, we see a dainty rise that maintains itself in low values. For the last ones, it is believed that the constant irradiation might have interfered with their ability to perform photosynthesis properly, as seen in the lower oxygen concentrations as well.

Regarding methane production rates under the 24-light regime, the unicellular strains CENA526 and CENA649 responded with higher continued peaks of production. All strains seemed to have a more uniform response for the rate of production, with CENA650 having higher peaks in the second 24-h with similar values when the lights were on, i.e. in the photoperiod regime; CENA622 have a 10-times increasement in the rate previously found, and both *A. elenkinii* presented lower values than before.

On the other hand, for the 24-h dark controls, it was expected to see lower or no methane production at all, if we consider that the process is related to light irradiation. For all strains tested, the oxygen concentrations had an instant drop and were not higher than 0.06 mmol, which is the lowest value detected in the whole experiment (Fig. 18). Once more, both *A. elenkinii* diverged from the others, having a strange momentary increase in the oxygen values. The unicellular CENA526 and CENA649 had the lowest oxygen values and were the most stable of them. Regarding methane concentrations and production, we unexpectedly still detected both from all strains. Fewer peaks of production are shown, however, curiously for some of them, the values are even higher than when it was under the photoperiod conditions.

Figure 19 is used as a reference for the proper functioning of the MIMS equipment, where the values are stable for all the periods evaluated. Also, water is being used as a control for the absence of contamination by any other organism in the incubation chamber as well.

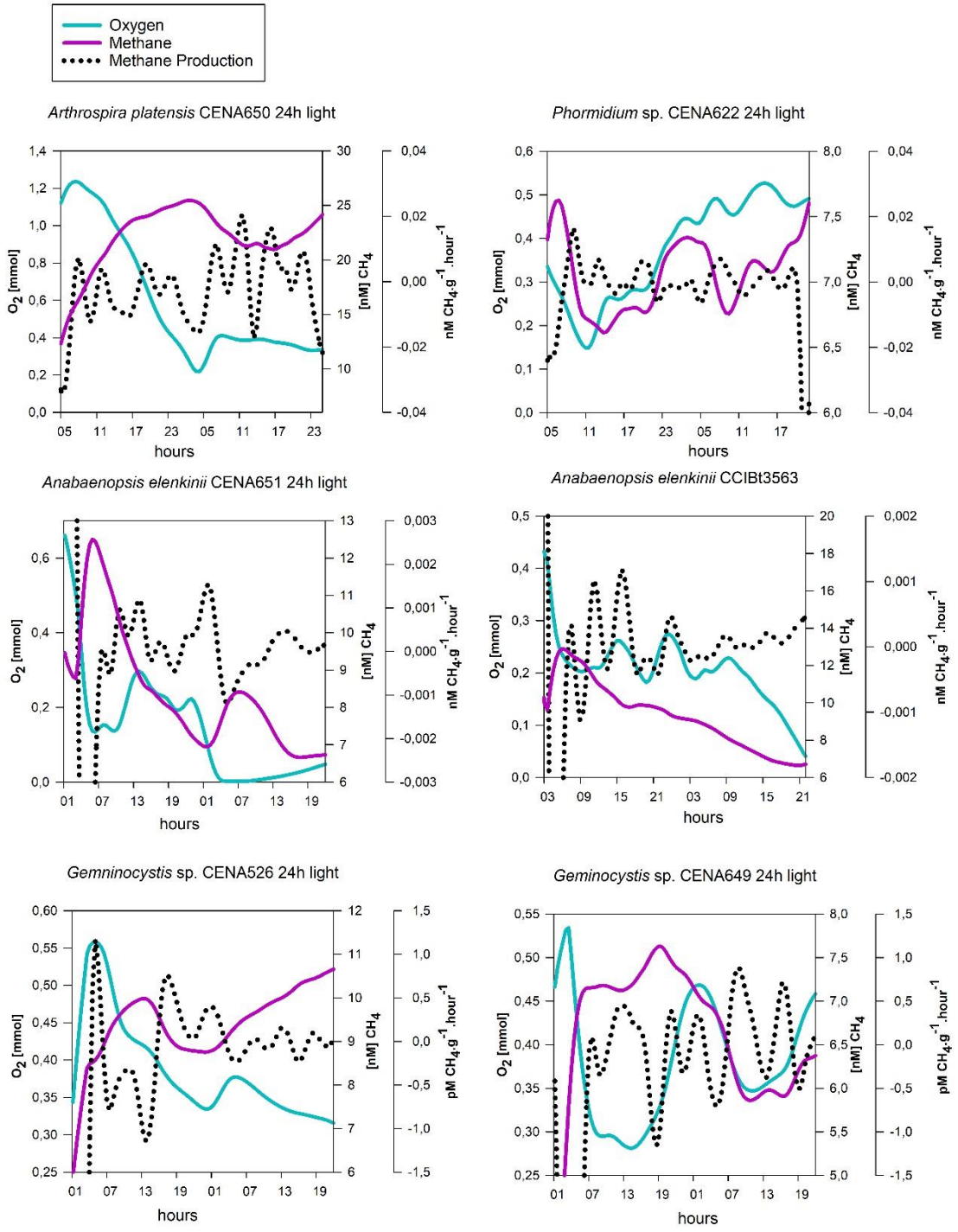

Figure 17. Continuous measurements of oxygen concentrations (blue), methane concentration (purple), and production rate (dotted black) under a 24-h light phase for control using MIMS.

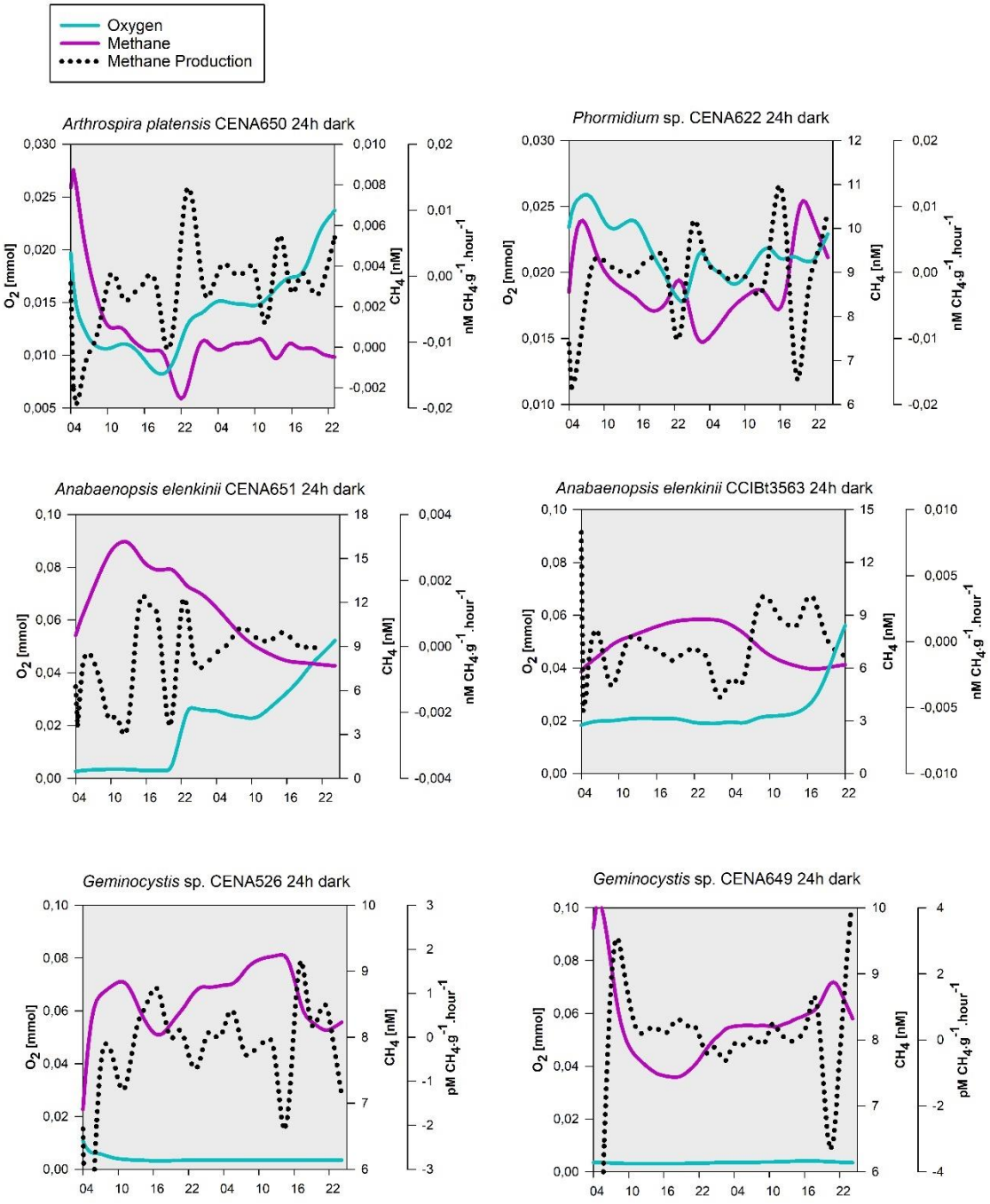

Figure 18. Continuous measurements of oxygen concentrations (blue), methane concentration (purple), and production rate (green) under a 24-h dark phase for control using MIMS.

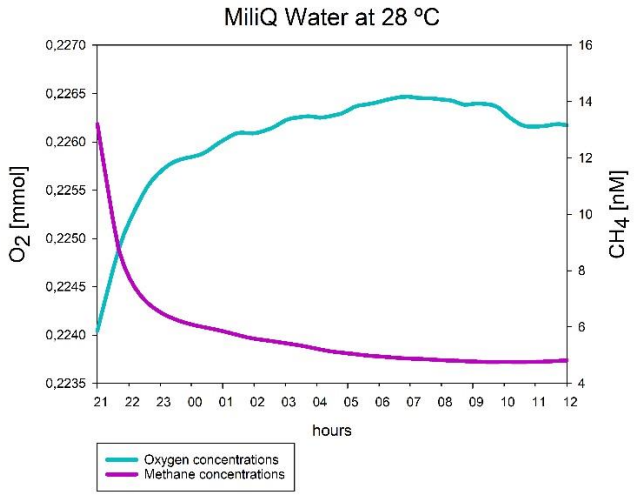

Figure 19. Continuous measurements of oxygen concentrations (blue) and methane concentration (purple) for standard control with MiliQ water using MIMS.

4.3.3. Experiment 2: Long-period methane continuous production using filamentous and unicellular strains

To improve interpretation, we selected one bloom-forming planktonic, one filamentous from the lakeshore, and one unicellular strain to be monitored for a longer period than 48 hours. *A.platensis* CENA650 was evaluated for about five days (Fig. 20), regarding their apparent methane production during both dark and light phases. Oxygen concentrations slopes follow what we expected to see, being higher during the light period. As we can see, the production curve shows that the initially high peaks occur when oxygen concentrations decrease, but this pattern is slightly changing when the cycles restart. We observed that the production of methane follows the oxygen concentration slops, but interestingly again there is an apparent lower peak of production in the dark phase as well. For this rerun, we hypothesize that the period for the cyanobacterial cultures to adapt to the photoperiod and temperature conditions is longer than expected, especially since they were previously incubated at very different culture settings.

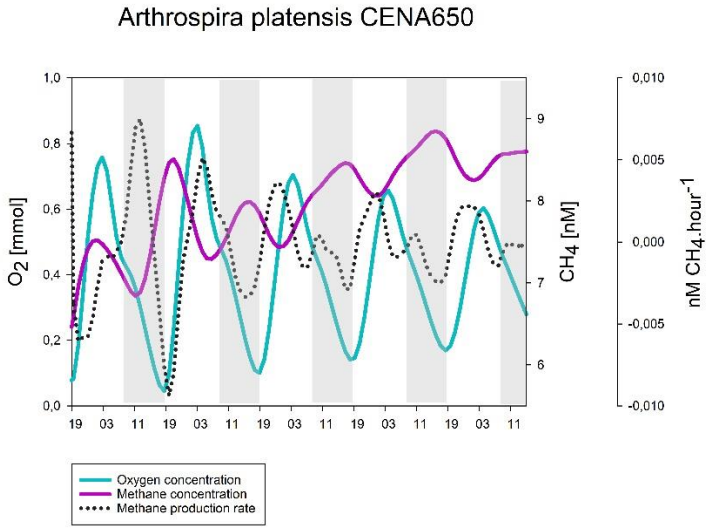

Figure 20. *Arthrospira platensis* CENA650 was used for continuous measurements of oxygen concentrations (blue), methane concentration (purple), and production rate (black dotted) at 14h light: 10h dark periods using a mass-inlet membrane spectrometry (MIMS).

As CENA622 showed quite odd results previously, it was rerun for a longer period than the other cultures to obtain some explanations. Similarly, to what we have seen for CENA650, there is a slight delay in the methane production to adjust to the light and oxygen periods. However, as time goes by it becomes clear that methane is being produced in presence of oxygen (Fig. 21). The range of methane production seems to be the same as we found previously, but there was for sure an adaptation issue.

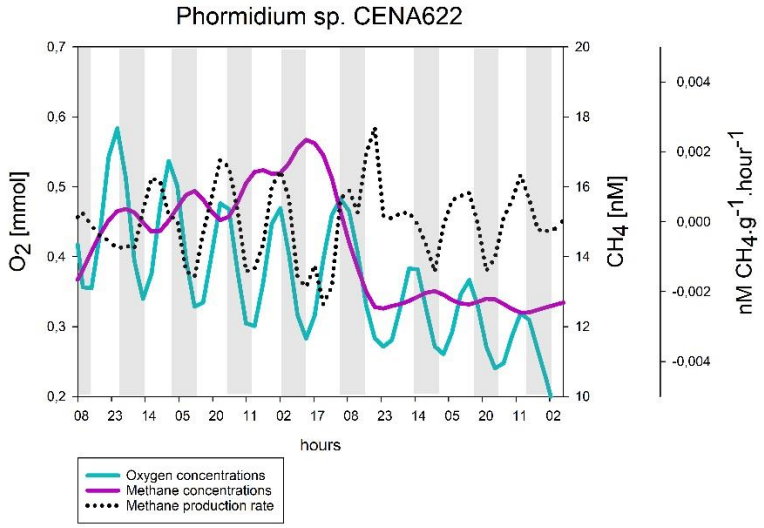

Figure 21. *Phormidium* sp. CENA622 was used for continuous measurements of oxygen concentrations (blue), methane concentration (purple), and production rate (black dotted) at 14h light: 10h dark periods using a mass-inlet membrane spectrometry (MIMS).

For CENA526, it is observable a similar pathway for oxygen and methane concentration measurement – oxygen accumulating during the light period, and methane during the dark one. However, when looking at the production rate of methane, the peaks all increase in the presence of

oxygen and decrease altogether. No methane peaks occur in the dark for this strain. For them, however, the cells were able to adapt their cycle more instantly than the filamentous ones (Fig. 22).

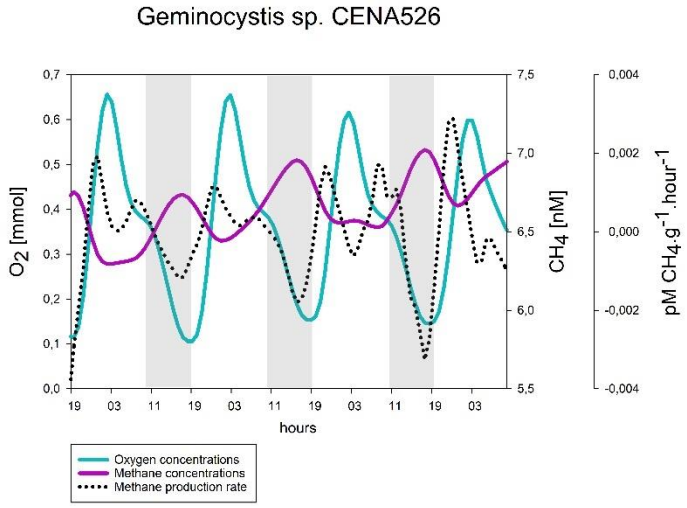

Figure 22. *Geminocystis* sp. CENA526 was used for continuous measurements of oxygen concentrations (blue), methane concentration (purple), and production rate (green) at 14h light: 10h dark periods using a mass-inlet membrane spectrometry (MIMS).

4.4. Discussion

To explore once more the involvement of cyanobacteria in methane release from the Brazilian soda lakes, we used isolates to be monitored *in vitro* and give more answers about their OMP contributions. In this study, we were able to identify methane production in both experiments tested for all cyanobacteria strains selected. Based on the recent work of Bižić *et al.*, (2020), we tested our tropical cyanobacteria in similar conditions to identify methane production. In their study, they found compelling evidence for methane production during photosynthesis, using carbon-labeled isotopes, and blocking different stages of photosynthesis to verify the effect on methane levels. Successfully, they brought a new perspective to OMP studies.

Figure 16 represents experiment 1, expressing our main interest in reassuring light-induced methane production. Although the cultures might present a certain divergence on the oxygen and methane concentrations slopes, all of them it was detected methane production under light irradiation and in the presence of the oxygen. We can follow all methane slopes in relation to the variations in O_2 concentration when the lights are turned on and off, which leads us to the photosynthetic activity of these organisms. During the oxygenic photosynthesis, oxygen is produced during carbon fixation. Therefore, the photosynthetic process might be connected to the production of methane by cyanobacteria, and other autotrophic organisms.

This pattern became stronger when we reran three cultures for a longer time period in experiment 2. All of them, bloom-forming, filamentous or unicellular revealed methane production in

the presence of oxygen (Fig. 20, 21, and 22). For CENA650 and CENA622, filamentous strains, it took a while for acclimating to the experimental conditions, but, eventually, oxygen and methane curves superpose. As it is reported in Bižić et al. (2020) methane concentrations produced by cyanobacteria are two times lower than the ones expected from Archaea via classical methanogenesis. Using MIMS, they found an average production rate of 1,000 to 100,000 μmol CH_4 h^{-1} g^{-1} for archaea isolates, while marine and freshwater cyanobacteria showed a mean range of 0.1 to 100 μmol CH_4 h^{-1} g^{-1}. However, if we consider that *A. platensis* can form massive blooms, the impact of this production could still be substantial.

Oxygenic photosynthesis involves the breakage of water molecules into electrons to generate ATP for energy. The electron-transport system is constituted of electron carriers (plastoquinone and plastocyanin), protein complexes photosystem I and II (PSI and PSII, respectively), and the cytochrome b_6f complex (Muramatsu & Hihara, 2012). PSII reflects the efficiency of the photosynthetic process acting on the presence of light to mediate the water oxidative reduction, generating O_2 as well (Nickelsen & Rengstl, 2013). Light-absorbing chlorophyll is part of the PSII, and its products come from the conversion of protochlorophyllide into chlorophyllide, the direct precursor of chlorophyll, which is done by the activity of the DPOR enzyme (or *chl* genes) (Nickelsen & Rengstl, 2013).

As mentioned in the literature, there are no records of the classical methanogenic marker *mcr*A for any other organisms than Archaea. Archaeal methanogenesis is a coordinated process, where methane is generated from specific precursors by the activity of the MCR enzyme. A proposed homolog has been mentioned in Bižić *et al.* (2020) as most of the enzymes required to convert organic compound into methane exists in many Bacteria, however, not the key one.

Perez-Coronel & Beman (2021) also performed evaluations on cyanobacterial production of methane induced by light and both were positively correlated, which was overall mentioned on chapter 1. In the same path that we looked at here, this close relation to light-induced they too searched for an influence of the photosynthetic process on the gas emissions. From transcriptomic analysis, they observed an overexpression for chlorophyll (DPOR) and bacteriochlorophyll (COR) biosynthetic reductases with a central role in the photosynthetic process. Therefore, they suggested potential genetic markers to unravel methane production in cyanobacteria in the presence of oxygen. In this case, it would fit our results, as it is not the nitrogenase from heterocyte, not being restricted to nitrogen-fixing strains. Morana *et al.* (2020) described that organic carbon (as acetate) and inorganic compound (as sulfur-bonded methionine) in the presence of light; however, only the methane coming from the fixation of inorganic carbon had relations to the photosynthetic activity and nitrogen fixation.

The adjustment to the circadian rhythms of the cultures to the experiment light regime might be closely related to methane production. Circadian rhythms consist of functions that oscillate in persistent rhythms when there is no time cues; able to reset the stage according to light or dark conditions; and temperature compensation of the circadian period (Cohen & Golden, 2015; Kondo &

Ishiura, 2000). In addition to feeding and defense, the fact that some cyanobacterial metabolisms are dependent on the alternation between day and night, the establishment of rhythms is fundamental for efficient behavior and enhancing their fitness (Leypunskiy et al., 2017). For example, unicellular cyanobacteria able to fix nitrogen use a temporal separation as a strategy to perform N fixation and photosynthesis, as nitrogenase is sensitive to oxygen when one is performed at night (dark conditions) and the other during the day (light conditions), respectively (Golden & Canales, 2003). So, the signals for photosynthesis are downregulated under dark and then reset by signals from the light again.

The resetting stage synchronizes the physiological process to biological events according to local time (Golden & Canales, 2003). *Synechococcus elongatus* is the species model for circadian oscillations studies in cyanobacteria and it was reported their environmental regulation of amino-acid uptake, which is regulated by the light/dark cycle, but, absent during constant light (Golden & Canales, 2003). During dark periods, it has been described for *S. elongatus* that most genes related to growth and photosynthesis are repressed (Leypunskiy et al., 2017). Therefore, when we look into the results of experiment 2 it becomes more evident how a regulation for the production rate can fit to the period of activity under the light.

Some studies have mentioned an association between methanogenic archaea and cyanobacteria as the explanation for methane in oxic regions (Table 2). For instance, the aggregates formed by the *Microcystis aeruginosa* cells would create an anoxic micro-environment, where archaea could perform methanogenesis through the classical pathway (Batista et al., 2019; Bogard et al., 2014; Grossart et al., 2011). Another possible symbiotic mechanism would be around the heterocyte, which is a differentiated cell where nitrogen fixation can occur. For this, heterocyte thicker cell wall isolates the nitrogenase enzyme activity - a process also non-tolerant to the presence of O_2. Here, the archaea would be attached to the heterocyte to take advantage of the, again, micro-anoxic zones (Grossart et al., 2011). At the same time, as nitrogen fixation releases H_2 as a subproduct from nitrogenase activity, these molecules could be consumed by the hydrogenotrophic archaea and generate methane (Berg et al., 2014). However, even though these are strong possibilities for methane related to cyanobacteria, it does not apply to our experiment as our amplicon screening for classical methanogenesis using the *mcr*A gene was negative for all samples tested after the experiment (Fig. 15).

To support our inferences, we performed tests using 24-h light and 24-h dark conditions to observe the changes in gas dynamics. At first, for the 24-h light assay (Fig. 17), the expected methane production would be higher and constant values of production, at least at the beginning of irradiation. However, in general, we had fluctuation peaks in methane production, but they were quite consistent through time. Oxygen was always present, even though it was possible to observe some decreases or intervals on the slopes. Shevela *et al.* (2020) used the MIMS to demonstrate that O_2 production is light-induced like we also observed; although, they identified photodamage to the cells related to the increase of light exposition period, consequently affecting O_2 production. They also realized an adaption period for O_2 production after rising the number of flashes of light over the cultures, followed

by a total dark period, and then back to the initial lower exposition. Therefore, that can explain the reduction, or even absence, of oxygen concentrations and methane production after the first 24-hours.

Possible photodamage effects are also observable in for both *A. elenkinii*, specially CCIBt3563 (Fig. 16). Right after the first period with the lights on, the peak of O_2 concentrations decreases instantaneously – here we had our first hint for the need for acclimation of the cultures to the assay conditions. *A. elenkinii* is a bloom-forming, N-fixing cyanobacterium with heterocyte cells. Until recently, there is no published information on the morphology and ecophysiology of CENA651; although, our analysis under the microscopy identified this strain as *A. elenkinii* as well, mainly differing from CCIBt3563 by sampling location at the Nhecolândia Pantanal (Delbaje et al., 2021).

Even though *Anabaenopsis* sp. can form blooms in soda lakes and other saline environments, they do not seem to be easily adapted to variations under laboratory conditions. For instance, Santos (2013) tested the effects of temperature and pH on *Anabaenopsis* sp. strains from the Pantanal soda lakes, and they saw significant results in their growth rate of them. It was quite interesting to realize that in the soda lakes temperature can reach between 30-40 ºC; however, the optimum temperature they better adjusted to in this experiment was about 25 ºC (Santos, 2013). In addition, as they also have gas vacuoles that allows them to change their position in the water column. Possibly, in the environment, *Anabaenopsis* are travelling through the water column to avoid the solar incidence damages. Therefore, the sensibility of *Anabaenopsis* sp. under controlled conditions might be a relevant factor to account for the physiology of both strains during experiment 1.

Figure 14 demonstrates the screening for methanotrophic organisms. Only three cultures tested positive for the presence of the gene marker *pmo*A – CCIBt3563, CENA651 and CENA622. Both CCIBt3563 and CENA651 have the lowest rates of methane production for all conditions tests. Moreover, CENA650, the filamentous strains without the presence of methanotrophic genes, presents higher methane production rates and average methane concentrations. Meaning that these organisms might be consuming the methane being produced here indeed, and decreasing the values registered. Even though, it is also important to consider that photoinhibition of methane consumers' activity can happen, and therefore, not allowing these organisms to severely impact the measurements (Tang et al., 2016).

When looking into the *A. platensis* CENA650 behavior in experiment 1, something quite interesting caught our attention. For the photoperiod condition, many peaks for methane production can be observed during the dark phase, with constancy in methane concentrations. This repeated itself on the longer runs for CENA650 and CENA622 at first as well. In this context, for CENA650 results we can implicate some explanations for the dark production: (1) reminiscent precursors from the photosynthetic activity; (2) ROS produced during the light phase; and (3) non-enzymatic methane production.

There are some possibilities to be explored when considering light-driven mechanisms, not only coupled with photosynthesis. McLeod *et al.* (2021) observed the relation of UV radiation to

methane (and other gases) formation from many phytoplanktonic cells, resulting from the photodegradation process and other stresses. Other factors such as the presence of phytoplankton, chlorophyll levels, biomass amount, light irradiation, and temperature have also been reported to stimulate or be associated with methane production in many environments (Donis et al., 2017; Klintzsch et al., 2019; Repeta et al., 2016; Tang et al., 2016).

Although not entirely unrevealed, a similar mechanism has been described for terrestrial plants that are also able to produce methane under stressful conditions in the environment. This emission can be part of the response mechanism against physical injuries, temperature fluctuations, water availability, UV radiation, and anaerobic situations, using organic carbon with methyl ($-CH_3$) or methoxyl ($-OCH_3$) groups from structural components, like pectin, lignin, and cellulose (Wang et al., 2013). The microalgae *Emiliania huxleyi* has also been reported to produce methane using bicarbonate as a carbon source, and after many processing steps, to generate methane at the end (Lenhart et al., 2015). This is close to the demethylations of organic compounds described previously in this thesis, methane production would be an instantaneous breakdown of organic molecules, releasing the functional groups (Wang et al., 2013).

Reactive oxygen species are ordinarily produced by any cell during respiration, cellular stress, and enzymatic reactions on the cytochrome, promoting oxidative stress. During excessive light absorption during photosynthesis, the energy can be transferred to other molecules leading to ROS formation (Montgomery, 2015). In this situation, ROS can act on lipids and implicate morphological damage (Montgomery, 2015). Normally, PSII can recover rapidly from photodamage in non-stressed living cells. However, because it is very sensitive to environmental changes, stronger light incidence can delay its repairment, modeling its activity faster than many other metabolisms (Nishiyama et al., 2005). As a consequence of photodamaging PSII proteins essential to repair, ROS is formed intracellularly, which might inhibit the repairment of PSII (Nishiyama et al., 2005). In the PSI, light stimulates the generation of ROS during electron transportation, from energy transference from excited pigments and iron-sulfur centers (Hsieh et al., 2014; Nishiyama et al., 2005). During excessive or strong irradiation of light, cyanobacteria tend to reduce the absorption of light, downregulation of PSI (Muramatsu & Hihara, 2012).

Considering the methane production peaks during the 24-h dark conditions (Fig. 18), other reactions might also be adding to the methane concentrations achieved here. Besides the likely microbial transformation of products from carbon fixation, chemical reactions resulting might be causing the generation of methane as well. Ernst *et al.* (2022) recently reported the formation of methane from the interaction between ROS produced by phototrophic, free iron, and methyl sulfides (like DMSO, dimethyl sulfoxide) instantaneously under Fenton-type conditions, without any specific enzymes. The Fenton-reaction produces toxic free radicals, where ROS might react with iron ions from Fe-S groups, $H_2O_2 + Fe^{2+} \rightarrow OH^- + \cdot OH + Fe^{3+}$ (Hsieh et al., 2014). The growth medium used for strains cultivation and the MIMS experiment is the Z8 medium (Kótai, 1972), which is known to be a

nutrient-rich medium for cyanobacteria and microalgae. In this medium, there is specifically 0.017 mM of iron and 31.51 mM of sulfur. If enough ROS is produced, it may stimulate the reaction and some amounts of methane are released as well.

These investigation using the Brazilian Pantanal strains proportionated new perspectives on methane production from the soda lakes, as they stem from a very unique biome and environmental conditions, where the cyanobacteria dominate the microbial community (Andreote et al., 2018; Genuário et al., 2018a). Moreover, as stated previously, the presence and intensity of cyanobacterial blooms have been pointed out as one of the main factors for elevated methane concentrations (Barbiero *et al.*, 2018). The soda lakes' halo-alkaline conditions favor many types of methanogenic processes, from the classical multistep process done by Archaea to light-driver photosynthetic precursors. Once more we bring another possible contribution of cyanobacteria to methane to be likely adding to the methane net from the Pantanal soda lakes. These should bring attention to eutrophicated environments, with one more reason to work on mitigating the bloom formation for good.

4.5. Conclusion

In this study, we concluded that:

- Cyanobacteria isolates from Brazilian soda lakes are able to produce methane in the presence of oxygen under light irradiation, with the rates depending closely on the biomass;
- The bloom-forming strain *Arthrospira platensis* is apparently producing methane under light and dark conditions, for yet not understood reasons;
- Similar methanogenic precursors, photopigments degradation, ROS activity, and non-enzymatic chemical reactions are some of the possible explanations for the results we achieved;
- There is a clear interference of the circadian cycle of cyanobacteria to their efficiency on producing methane.

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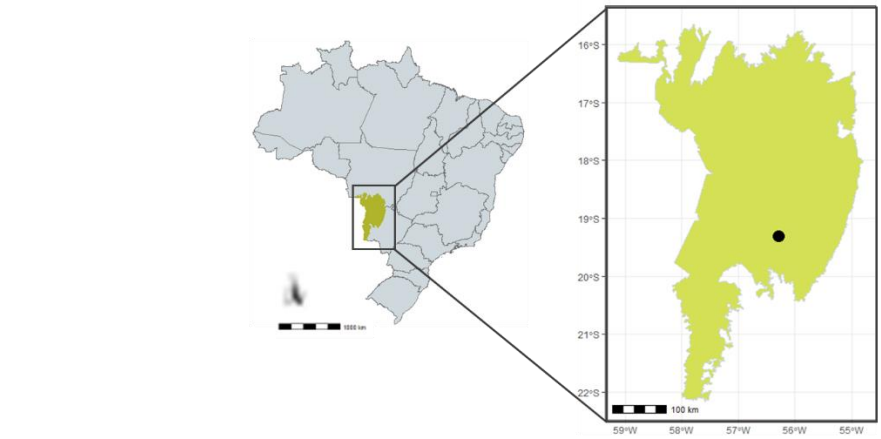
5. OVERALL CONCLUSION

In conclusion, our intentions to investigate oxic methane production pathways on the Brazilian Pantanal soda lakes are considered satisfactorily heeded, including the cyanobacterial role on this process. Although we highlight again that cyanobacteria might not be a major direct contributor on our shallow soda lakes, there are still many possibilities for them to be related to the final methane emissions. Our general overview guided the study on potential metabolisms that could be further explored on metagenomics data, such as the methylphosphonate demethylation pathway, or 'C-P lyase'. Even though we lacked on methane flux or concentrations data, we were still able to identify the 'C-P lyase' role on the microbial communities of these lakes, relating the drivers for phosphonate consumption to aid our inferences on the potential for this OMP to be actively happening. For our initial hypothesis, MPn demethylation cannot be confirmed to be performed in high rates by cyanobacteria, as the gene is only present in a non-bloom forming strain.On the other hand, we were able to demonstrate that the strains, including those apt of blooming, are capable of directly produce methane. Among many of the explanations we explored, generating methane precursor or reactive oxygen species during photosynthesis; non-enzymatic pathways interactions; and association with other microbes capable of methanogenesis or OMP, are the strongest ones. A very important take-home message from this study is, when investigating methane emissions, it is important to consider more than the classical known pathway. Overall, it is acceptable to define general major sources for methane production. However, specific points need to be taken into account for each environment, namely its microbial community, physical and chemical features, spatial and seasonal variables, because many sources can be acting simmultaneosly in a complex and not so straightforward way, such as observed in the Pantanal soda lakes

APPENDICES

APPENDIX A. Experimental area. Brazilian Pantanal and the soda lakes from the subregion Nhecolândia, located at the "São Roque" Farmland area (19º18'31.739"S, 56º17'26.356"W). The map was generated using the packages geobr and ggplot2 in R 4.2.0. Photos by Thierry A. Pellegrinetti.



04SR 06SR 07SR 08SR 09SR

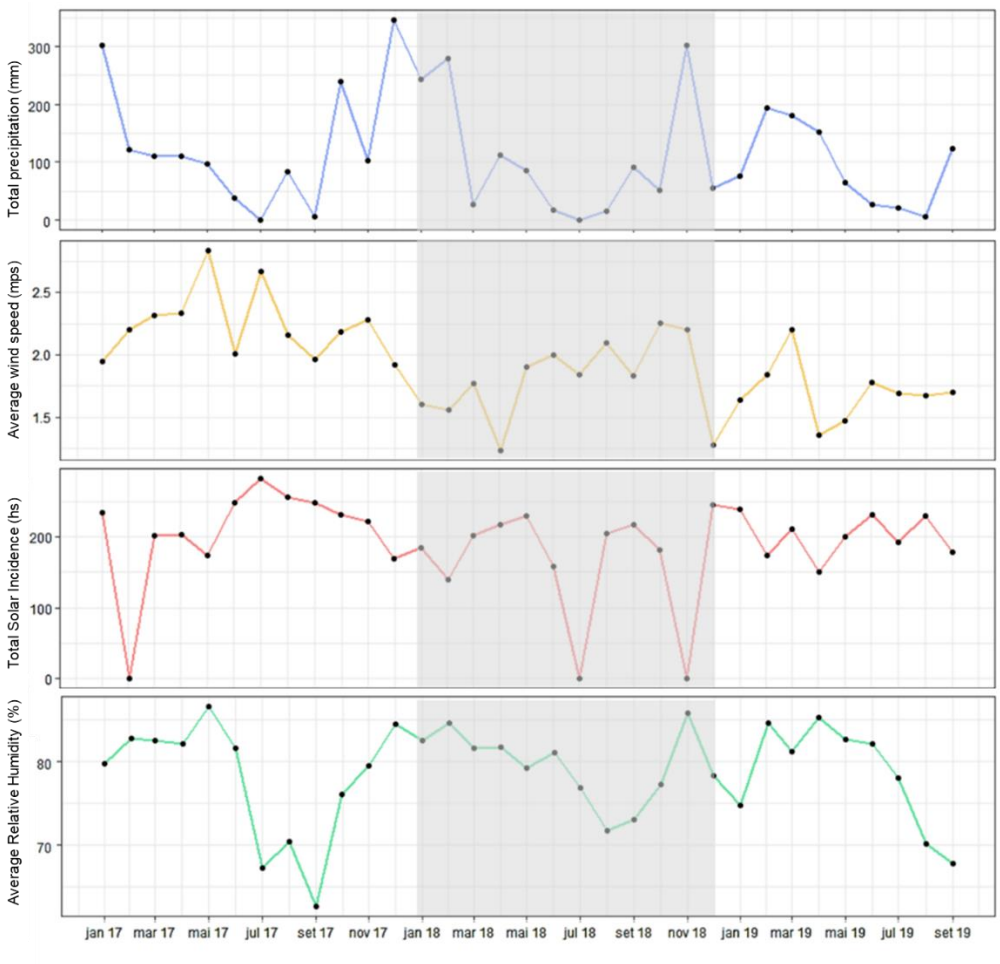
APPENDIX B. Soda lakes characterization (mean ± standard deviation) during 2017 and 2019 sampling moments.

Parameter	Year	04SR	06SR	07SR	08SR	09SR
Physical						
Area (km^2)	2017	56,000	9,000	179,000	1,500	160,000
	2019	125,000	245,000	192,000	98,000	380,000
Depth (cm)	2017	41	20	56	10	40
	2019	78.33	60	103.33	50	50
Water volume (m^3)	2017	22,960	1,800	100,240	150	64,000
	2019	285,416	14,700	153,600	49,000	190,000
Water temperature (°C)	2017	26.43	37.66	27.25	35.05	38.99
		±0.11	±0.16	±0.01	±0.13	±0.02
	2019	26.32	25.30	27.46	27.68	27.18
		±0.41	±0.14	±0.14	±0.21	±0.24
Chemical						
Alkalinity ($mmol.L^{-1}$)	2017	97.49	66.95	8.03	200.02	66.61
		±3.81	±2.72	±0.35	±8.8	±3.65
	2019	16.46	5.70	3.45	11.73	11.66
		±1.19	±0.18	±0.1	±0.25	±0.12
Electric conductivity	2017	16295	17990	1777	39257	18879
($\mu S\ cm^{-1}$)		±557.65	±65.51	±0	±194.44	±51.03
	2019	3202.5	1275	694.75	2552.5	2762.5
		±62.91	±31.09	±0.95	±22.17	±9.57
Dissolved organic carbon ($mmol\ L^{-1}$)	2017	0.92	0.30	0.07	5.32	0.69
		±0.17	±0.02	±0.005	±0.18	±0.06
	2019	252.9	40.01	36.53	141.13	130.40
		±14.01	±2.00	±16.89	±18.02	±7.62
Iron (µM)	2017	2.27	3.95	15.75	3.81	1.54
		±0.17	±1.46	±1.28	±0.06	±0.41
	2019	2.88	1380.22	0.48	1.52	3.10
		±0.40	±94.77	±0.10	±0.59	±3.05
Nitrate (µM)	2017	12.55	13.66	0.90	3.65	20.27
		±0.11	±1.29	±0.18	±1.09	±0.75
	2019	5.61	14.35	0.25	2.85	1.61
		±2.38	±1.33	±0.25	±0.28	±0.73
Ortho-phosphate (µM)	2017	885.42	915.18	4.38	636.22	64.37
		±42.70	±118.89	±0.57	±39.20	±22.89
	2019	161.20	129.44	0.24	47.29	19.25
		±0.10	±11.22	±0.06	±2.88	±0.50
Dissolved oxygen (%)	2017	28.73	84.86	84.90	68.03	174.70
	2019	36.90	86.66	140.56	369.80	114.43

pH	2017	10.01	9.69	8.55	10.07	9.91
	2019	10.26	9.45	9.04	9.90	9.99
TN:TP ratio (µM)	2017	18.65	4.16	98.47	35.69	90.93
		±0.19	±0.37	±7.04	±1.05	±5.61
	2019	8.84	3.07	1378.76	18.93	44.26
		±1.02	±0.45	±1791.31	±5.97	±4.83
Salinity	2017	11.10	9.71	0.98	25.12	12.24
(g L^{-1})		±1.74	±0.62	±0.15	±2.14	±0.67
	2019	2.39	0.89	0.42	1.56	2.20
		±0.10	±0.13	±0.06	±0.08	±0.27
Limnological						
Chlorophyll-*a* (µg L^{-1})	2017	2046.17	0.01	0.22	11348.04	332.24
		±958.5	±0	±0.1	±1476.2	±60.66
	2019	4123.01	22.93	60.86	821.01	178.87
		±361.48	±0.83	±3.3	±193.29	±59.75
Cyanobacterial bloom	2017	P	A	A	P	P
	2019	P	A	A	P	P

P: presence; A: Absence.

APPENDIX C. Meteorological data at the experimental area from 2017, 2018 and 2019. Retrieved from: BDMEP/INMET.



APPENDIX D. Total assigned reads on the metagenomics data using the function compare on MEGAN6.

Lakes	Total reads assigned
	3,761,499
04SR	4,310,704
	3,641,861
	2,988,590
06SR	4,101,728
	2,573,701
	2,973,586
07SR	3,390,984
	3,948,206
	4,285,311
08SR	4,439,976
	3,236,442

APPENDIX E. Raw data for dissolved methane concentrations

Sampling point	**SR04**	**SR06**	**SR07**	**SR08**
Média ATM	0.03	0.03	0.02	0.02
P1	11.65	0.23	2.58	11.65
P1	15.52	0.03	4.29	12.00
P1	14.54	0.02	5.07	11.95
P2	11.95	0.02	5.33	10.34
P2	13.13	0.06	1.37	13.94
P2	10.52	0.14	3.45	11.05
P3	14.00	0.01	2.66	17.43
P3	17.80	0.01	1.73	19.80
P3	14.90	0.02	4.70	20.00
P4	13.17	0.01	3.04	18.59
P4	11.85	0.01	4.29	21.68
P4	13.51	0.03	3.07	17.83
Mean ± SD	13.55 ± 1.58	0.05 ± 0.04	3.46 ± 0.39	15.52 ± 4,27

APPENDIX F. Cyanobacteria genomes from GenBank NCBI used for the phylogenetic tree construction.

Species	Strain ID	Order; Family	Morphology	Location	Environment	N-fixation
Leptolyngbya sp.	BC1307	Synechococcales; Leptolynbyaceae	Filamentous; mats	Antarctica	Lake	Non-heterocyte; N-fixing only during the day.
	Heron Island J			Australia		
Phormidesmis priestleyi	Ana_ITZX	Synechococcales; Leptolynbyaceae	Filamentous; mats	Washington, USA	Saline lake	N-fix genes.
Multispecies_cyanobacteria						
Filamentous_cyanobacteria	CCP5		Filamentous	Massachussets; USA	Salt marsh	
Limnoraphis robusta	CS-951	Oscillatoriales; Oscillatoriaceae	Filamentous	UK	Brackish ditch	N-fix geenes; non-heterocyte.
Lyngbya sp	PCC 8106	Oscillatoriales; Oscillatoriaceae	Filamentous	Germany	Intertidal zone	
Lyngbya aestuarii	BL J			Mexico	Marine microbial intertidal zone	Non-heterocyte; N-fix genes
Anabaena cylindrica	PCC 7122	Nostocales; Nostocaceae;	Filamentous	UK	Water, most likely pond	Heterocyte
Calothrix brevíssima	NIES 22	Nostocales; Calotrichaceae	Filamentous	Palau	Freshwater; Soil	N-fix
Trichormus variabilis	NIES 23	Nostocales; Nostocaceae	Filamentous	Unkown		Heterocyte
Raphidiopsis curvata	NIES 932	Nostocales; Aphanizomenonaceae	Filamentous	Japan, Tokio	Freshwater, pond	N-fix
Fischerella thermalis	CCMEE 5330	Nostocales; Hapalosiphonaceae	Filamentous	Japan	Hot spring	Heterocyte
Synechococcus sp.	JA23Ba213	Synechococcales; Synechococcaceae;	Unicelular	USA	Hot spring microbial mats	N-fix genes
Scytonema hofmannii		Nostocales; Scytonemataceae;	Filamentous	UK	Limestone cave	Heterocyte
Scytonema hofmannii	PCC 7110					
Unicelular cyanobacterium	SU3					
Cyanothece sp	PCC 8802	Chroococcales; Aphanothecaceae;	Unicelular	Taiwan	Rice field	Diazothropic
	PCC 8801					