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Ecophysiological investigation of the cyanobacteria *Anabaenopsis elenkinii* and *Limnospira platensis*: predominant species in saline/alkaline lakes of the Pantanal Wetland

Investigações ecofisiológicas das cianobactérias *Anabaenopsis elenkinii* e *Limnospira platensis*: espécies predominantes em lagoas salinas/alcalinas do Pantanal

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Abstract: Aim: In this study, we investigated the distribution of *Anabaenopsis elenkinii* and *Limnospira platensis* in the saline-alkaline lakes of Nhecolândia (Pantanal wetland) and evaluated the impact of pH, temperature, and nitrogen on their growth and development to understand their ecological responses, showing insights into their ecophysiology in both cultured and natural environments. **Methods:** Both species were collected in the subsurface, using a plastic bottle (200 mL) and the parameters temperature, conductivity, and pH were measured *in situ*. From these samples, the strains *A. elenkinii* CCIBt1059 and *L. platensis* CCIBt3335 were isolated and underwent six different cultivation treatments, in triplicate, during 30 days, with daily cell count, photoperiod of 12-12 hours of light-dark, and light intensity between 80-100 µmol photons $m^{-2}.s^{-1}$, using BG-11 modified medium, as follows: nitrate concentration experiments were performed with a 750 $mg.L^{-1}$ $NaNO_3$ (50%) and a nitrogen-free condition (0%) (T1 vs T2); temperature experiments were performed with 30 °C and 35 °C (T3 vs T4); pH experiments with 10.5 and 7.0 (T5 vs T6). The adopted control conditions were BG-11m medium (3% $NaNO_3$, 45 $mg.L^{-1}$), pH adjusted to 9.5, and temperature set at 25 °C. **Results:** We have found that the treatment with the highest nitrogen availability (T1), pH of 9.5, and a temperature of 25 °C, provides the most favorable conditions for the growth of both studied species. In nature, *A. elenkinii* occurred predominantly between pH 9.04 to 10.4 (average 9.8) and *L. platensis* at pH 9.22 to 10.23 (mean 9.9), highlighting the alkaliphilic nature of these species. Furthermore, we observed that temperature

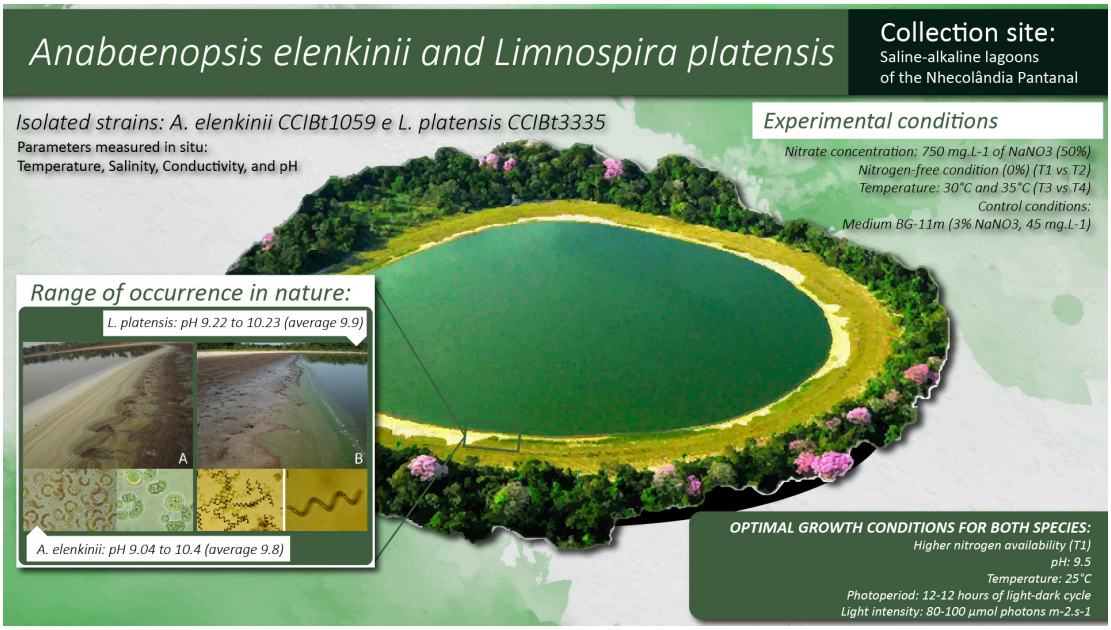
influences the frequency of heterocyte formation in *A. elenkinii*. At elevated temperatures (30 and 35 °C), the frequency of heterocytes was higher compared to 25 °C during the exponential growth phase, indicating that increased heterocyte formation is a strategy in response to temperature stress. **Conclusions:** This research provides valuable insights into the ecological aspects and optimization of the cultivation of the two species studied, which hold ecological significance to saline lakes. Further studies are recommended to explore their potential biotechnological applications.

Keywords: cyanobacterial physiology, cyanobacterial growth, heterocyte formation, nitrogen availability, tropical environment.

Resumo: Objetivo: Neste estudo, investigamos a distribuição de *Anabaenopsis elenkinii* e *Limnospira platensis* nas lagoas salino-alcalinas do Pantanal da Nhecolândia e avaliamos o impacto do pH, temperatura e nitrogênio no crescimento e desenvolvimento dessas espécies para entender suas respostas ecológicas, mostrando *insights* em sua ecofisiologia em ambientes cultivados e naturais. **Métodos:** Ambas as espécies foram coletadas em garrafas plástica (200 mL) e os parâmetros temperatura, condutividade e pH foram medidos *in situ*. A partir dessas amostras, as cepas *A. elenkinii* CCIBt1059 e *L. platensis* CCIBt3335 foram isoladas e submetidas a seis diferentes tratamentos de cultivo, em triplica, durante 30 dias, com contagem de células diárias, fotoperíodo 12-12 horas de claro-escuro e intensidade luminosa 80-100 µmol fótons $m^{-2}.s^{-1}$, utilizando meio BG-11 modificado, a saber: experimentos de concentração de nitrato foram realizados com $NaNO_3$ 750 $mg.L^{-1}$ (50%) e uma condição livre de nitrogênio (0%) (T1 vs T2); experimentos de temperatura foram realizados com 30 °C e 35 °C (T3 vs T4); e experimentos de pH com 10,5 e 7,0 (T5 vs T6). As condições de controle adotadas foram meio BG-11m (3% $NaNO_3$, 45 $mg.L^{-1}$), pH ajustado para 9,5 e temperatura regulada para 25 °C. **Resultados:** Descobrimos que o tratamento com maior disponibilidade de nitrogênio (T1), pH 9,5, temperatura 25 °C, proporciona as condições mais favoráveis para o crescimento de ambas as espécies estudadas. Na natureza, *A. elenkinii* ocorreu predominantemente entre pH 9,04 a 10,4 (média 9,8) e *L. platensis* em pH 9,22 a 10,23 (média 9,9), ressaltando a natureza alcalifílica dessas espécies. Além disso, observamos que a temperatura influencia a frequência de formação de heterócitos em *A. elenkinii*. Em temperaturas elevadas (30 e 35 °C), a frequência de heterócitos foi maior em comparação com 25 °C durante a fase de crescimento exponencial, indicando que o aumento da formação de heterócitos é uma estratégia em resposta ao estresse térmico. **Conclusões:** Esta pesquisa fornece informações valiosas sobre os aspectos ecológicos e otimização do cultivo das duas espécies estudadas, que possuem importância ecológica para as lagoas salinas. Mais estudos são recomendados para explorar suas potenciais aplicações biotecnológicas.

Palavras-chave: fisiologia de cianobactérias, crescimento cianobacteriano, formação de heterócitos, disponibilidade de nitrogênio, ambiente tropical.

Graphical Abstract



1. Introduction

The Pantanal is the largest tropical wetland on the planet, spanning more than 150,000 km^2 and located in the central part of South America (Magalhães Neto & Evangelista 2022). The Nhecolândia sub-region of Pantanal Biome (Brazil) stands out as a highly complex region with a dense hydrographic network. It is located in Mato Grosso do Sul State and it is bordered to the north and south by the Taquari and Negro Rivers, to the east by the escarpment of the Serra de Maracajú, and to the west by the Paraguay River (Allem & Vals, 1987; Fernandes et al., 1996). With an estimated area of 26,921 km^2, Nhecolândia occupies 19.5% of the total area of the Pantanal, representing one of the largest sub-regions of this biome (Silva & Abdon 1998).

The main characteristic of the Pantanal da Nhecolândia is the presence of thousands of shallow lagoons (with depth not exceeding 2 m), predominantly circular, ranging from 50m to 2-3 km in their longest dimension. These lagoons, according to their distinct limnological characteristics, are regionally referred to as "baías" (bays – typical freshwater lagoons, with macrophytes and fishes), "salitradas" (salt marshes – lagoons with intermediated aspects when compared to salines and bays), and "salinas" (salines – typical brackish lagoons, without macrophytes and fishes) (Calheiros & Oliveira 1999). Fernandes (2007) estimated the presence of 9.324 lagoons in the Lower Nhecolândia, with 84% of them classified as bays (7,832) and 16% as salines (1,492). This high concentration of peculiar lagoons makes the landscape unique in the world. The salines of Nhecolândia are alkaline lagoons with brackish water and exhibit varied colors, ranging from greenish and bluish to brownish. They are dominated by the cyanobacteria *Anabaenopsis elenkinii* Miller and *Limnospira platensis* (Gomont) Santos & Hentschke (Santos et al., 2018; Santos & Sant'Anna, 2010 - cited as *Arthrospira platensis*).

A. elenkinii is a heterocytous cyanobacterium that occurs in brackish and alkaline waters and is recognized as an alkaliphilic species (Iteman et al., 2002; Santos et al., 2011, 2018). This species is frequently documented as part of the phytoplankton community in alkaline-saline lakes in East Africa, often in high densities and associated with *Arthrospira* spp. (Ballot et al., 2008). In 2007, massive mortality of birds, mainly Flamingos (*Phoeniconaias minor* Goeffroy), associated with toxic blooms of *Anabaenopsis* spp. and *Limnospira* (*Arthrospira*) *fusiformis* Nowicka-Krawczyk et al., was documented in a shallow lake in Greece (Lake Koronia) (Moustaka-Gouni, 2007). Other authors have recognized the production of microcystins by *Anabaenopsis* species (Lanaras & Cook, 1994).

Limnospira platensis is a cyanobacterial species characterized by typical coiled and homocytous filaments. This species was first described as "*Arthrospira platensis* Gomont", but was recently combined into the genus *Limnospira* by Santos et al. (2023), who considered molecular, morphological, and ecological analyses. Before that, the genus *Arthrospira sensu* Gomont was previously divided into two taxa: the benthic type species *Arthrospira jenneri* Stizenberger ex Gomont, which lacks gas vesicles, and the planktonic species, which now compose the new genus *Limnospira* (Nowicka-Krawczyk et al., 2019). Both genera exhibit spiraled trichomes with visible septa, which is the main morphological difference from *Spirulina* Turpin ex Gomont.

The *Limnospira* species, mainly *L. platensis* and *L. maxima* (Setchell & N.L.Gardner) Nowicka-Krawczyk, Mühlsteinová & Hauer are widely used as a commercial product (named "Spirulina") with high nutritional value, serving as a raw material for the food, chemical, and pharmaceutical industries (Lopes et al., 2022). They present therapeutic properties in the treatment of diseases such as cancer, hypercholesterolemia, and atherosclerosis (Colla et al., 2007). Additionally, these species produce phenolic compounds, antioxidants, antivirals, and anti-inflammatory agents (Piñero Estrada et al., 2001; Lopes et al., 2022). Regarding to *L. platensis*, its use as a dietary supplement for humans and animals is recommended by the FAO (Food and Agriculture Organization) due to its superior protein amino acid composition, with about 65-70% of the dry weight consisting of proteins (United Nations, 2005). In Brazil, the distribution of this species appears to be restricted to the Nhecolândia salines, and studies on it are scarce, most of them using strains obtained from the culture collection of the University of Texas (*Ar. platensis* UTEX 1926) (Bezerra et al., 2008).

In this study, we aim to isolate and characterize Brazilian strains of *Limnospira platensis* and *Anabaenopsis elenkinii*, which are ecologically significant species. Moreover, our goals are to enhance taxonomic understanding and gain insights into their ecophysiology in both cultured and natural environments. We specifically investigate their distribution in the saline-alkaline lakes of Pantanal da Nhecolândia and evaluate the impact

of pH, temperature, and nitrogen on their growth and development to understand their ecological responses.

2. Material and Methods

2.1. Sampling and strain isolation

To study the distribution of *L. platensis* and *A. elenkinii* in the Pantanal da Nhecolândia, Brazil, twenty-seven water samples were collected from lakes in this region during both the dry and rainy seasons from 2004 to 2012. For each sample, one part was fixed in the field with a 4% formaldehyde solution for preservation, while the other part was kept without any preservative for the study of living and cultured material. These preserved samples were then deposited in the State Scientific Herbarium 'Maria Eneyda P. Kauffmann Fidalgo' (SP) at the Instituto de Pesquisas Ambientais (formerly known as Instituto de Botânica) (Figure 1, Table 1). The collection method involved immersing a plastic bottle (200 mL) in the subsurface of the margin and central region of the lakes. The parameters temperature, conductivity, and pH were measured *in situ* with a WTW 340i probe (Table 1).

The strains selected for the physiological studies were sampled at Salina do Meio lagoon as described above. From these samples, the strains *L. platensis* CCIBt3335 and *A. elenkinii* CCIBt1059 were isolated in the laboratory using the microscope and a micropipette to transfer individuals to tubes with BG-11 (Stanier et al., 1971) modified with a nitrogen concentration of 3% ($NaNO_3$, 45 $mg.L^{-1}$) and pH adjusted to 9.5 with addition NaOH 1M (BG-11m) which aimed to mimic the natural conditions of the sampling site. Following isolation, the strains were added to the Culture Collection of the Institute of Botany of São Paulo (CCIBt) and maintained in triplicate under controlled conditions: a temperature of 23 ± 1 °C, light intensity of 40-50 µmol $photons.m^{-2}.s^{-1}$, and a photoperiod of 14-10 hours light-dark.

2.2. Preparation of experiments

For both strains, the steps to prepare the experiments were: a) 5 mL of inoculum were transferred to an Erlenmeyer flask containing 50 mL of BG-11m medium and kept in a constant rotation of 70 rpm (revolutions per minute) for 7 to 10 days; b) After the culture growth, 50 mL of this inoculum were transferred to 500 mL of medium, maintained at 70 rpm for 10 to 12 days; c) From the obtained 500 mL inoculum (10^5-10^6 $cells.mL^{-1}$), 50 mL were transferred to a 1,000 mL Erlenmeyer flask with 500 mL and then transferred to the culture chambers without aeration or agitation to perform the experiments. The conditions in the chambers were set to a photoperiod of 12-12 hours light-dark, light intensity of 80-100 µmol $photons.m^{-2}.s^{-1}$ (measured by a Li-COR model LI-250), and temperature of 25 °C, controlled by a thermostat in a camera type B.O.D. (model 347-CDG Fanem).

2.3. Strains growth analysis and statistics

The growth experiments were conducted over 30 days and in triplicate, based on daily cell counts (from a 0.7 mL sample fixed with the addition of 0.7 mL 4% formaldehyde solution, resulting in a 2× dilution factor) using a Fuchs-Rosenthal counting chamber. To assess the growth of the strains under different treatments, a Multilevel Factorial Design (Montgomery 2009) $3^1 \times 2^1$ was employed, involving 3 variables (nitrate concentration, temperature, and pH) at two levels of variation, resulting in

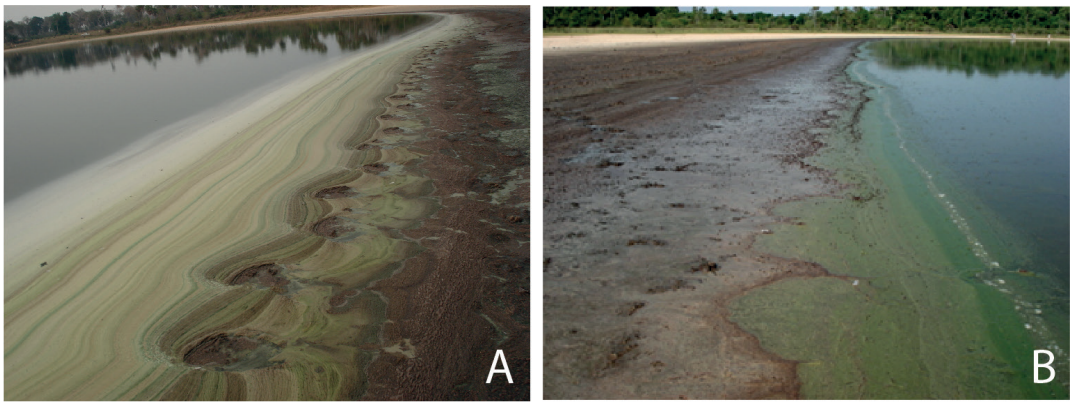


Figure 1. General view of saline lagoons from Pantanal da Nhecolândia. (A) Bloom of *Anabaenopsis elenkinii*; (B) Bloom of *Limnospira platensis*.

Table 1. Geographical position, herbarium voucher, water physicochemical characteristics, and dominant cyanobacterial species in the lagoons of Pantanal da Nhecolândia.

Lagoon	Herbarium voucher	Geographical position	Sampling date	pH	Electric conductivity (µS/cm)	Temp. (°C)	Dominant species
Salina do Meio	SP400654	18°58'29"S, 56°38'47"W	19/08/2009	9.47	10,600	32.5	*A. elenkinii*, *L. platensis*
Salina do Meio	SP427290	18°58'29"S, 56°38'47"W	27/10/2011	9.89	4,060	29.7	*A. elenkinii*, *L. platensis*
Salina do Meio	SP427740	18°58'29"S, 56°38'47"W	06/05/2012	9.64	8,770	-	*A. elenkinii*, *L. platensis*
Salina do Meio	SP390917	18°58'29"S, 56°38'47"W	25/09/2005	9.85	19,020	23.3	*A. elenkinii*, *L. platensis*
Salina do Meio	SP390919	18°58'29"S, 56°38'47"W	21/04/2006	10.16	2,870	33.3	*A. elenkinii*, *L. platensis*
Salina do Meio	SP390922	18°58'29"S, 56°38'47"W	28/08/2006	10.09	12,070	24.7	*A. elenkinii*, *L. platensis*
Salina do Meio	SP390927	18°58'29"S, 56°38'47"W	04/05/2007	10.19	3,890	31.0	*A. elenkinii*, *L. platensis*
*Salina do Meio	SP390924	18°58'29"S, 56°38'47"W	16/11/2006	10.4	4,380	36.1	*A. elenkinii*
*Salina do Meio	SP390916	18°58'29"S, 56°38'47"W	28/06/2005	9.85	6,850	21.7	*A. elenkinii*
*Salina do Meio	SP390913	18°58'29"S, 56°38'47"W	08/05/2005	9.04	5,500	25.0	*A. elenkinii*
*Salina do Meio	SP390910	18°58'29"S, 56°38'47"W	14/10/2004	9.88	9,030	24.2	*A. elenkinii*
*Salina do Meio	SP390907	18°58'29"S, 56°38'47"W	10/05/2004	9.6	-	34.2	*A. elenkinii*
Salina da Reserva	SP400842	18°57'35"S, 56°37'18"W	09/05/2005	-	2,380	22.9	*A. elenkinii*, *L. platensis*
Salina da Reserva	SP401692	18°57'35"S, 56°37'18"W	19/08/2009	9.68	11,040	34.1	*A. elenkinii*, *L. platensis*
Salina da Reserva	SP427741	18°57'35"S, 56°37'18"W	06/05/2012	10.1	5,435	-	*A. elenkinii*, *L. platensis*
Salina da Ponta	SP400843	18°58'56"S, 56°39'33"W	25/09/2005	9.9	5,790	23.8	*A. elenkinii*, *L. platensis*
Salina da Ponta	SP400845	18°58'56"S, 56°39'33"W	22/04/2006	9.8	864	32.8	*A. elenkinii*, *L. platensis*
Salina da Ponta	SP400487	18°58'56"S, 56°39'33"W	28/08/2006	9.8	8,970	22.8	*A. elenkinii*, *L. platensis*
Salina da Ponta	SP400849	18°58'56"S, 56°39'33"W	17/11/2006	9.9	716	30.8	*A. elenkinii*, *L. platensis*
Salina da Ponta	SP401691	18°58'56"S, 56°39'33"W	19/08/2009	9.22	8,130	30.4	*A. elenkinii*, *L. platensis*
Salina Pedra do Sol	SP427742	19°10'36"S, 56°57'44"W	26/08/2006	10.06	2,100	28.0	*A. elenkinii*, *L. platensis*
Salina Pedra do Sol	SP427743	19°10'36"S, 56°57'44"W	16/11/2006	10.23	12,200	32.0	*A. elenkinii*, *L. platensis*
Salina Pantanal	SP427747	18°55'40"S, 56°33'03"W	16/08/2009	-	-	-	*L. platensis*, *A. elenkinii*
*Salina Centenário	SP427277	19°28'39"S, 56°03'37"W	23/10/2011	9.64	1,743	26.1	*A. elenkinii*
*Salina da Botina	SP427278	19°26'38"S, 56°03'41"W	23/10/2011	9.18	1,105	30.6	*A. elenkinii*
Salitrada Campo Dora	SP390925	18°58'05"S, 56°38'58"W	16/11/2006	8.42	1,852	32.0	*L. platensis*, *Spirulina subsalsa*, *A. elenkinii*
*Baía da Sede Nhumirim	SP390926	18°59'37" S, 56°37'14" W	16/11/2006	7.84	2,020	35.1	Just some trichomes of *A. elenkinii*

**Only Anabaenopsis elenkinii* (without *Limnospira platensis* in the environmental sample). – Data not obtained.

6 cultivation conditions in addition to the control (C) condition (n=21; Table 2), as follows: nitrate concentration experiments were performed with a 750 mg.L^{-1} $NaNO_3$ (50%) and a nitrogen-free condition (0%) (T1 vs T2); temperature experiments were performed with 30 °C and 35 °C (T3 vs T4); pH experiments with 10.5 and 7 (T5 vs T6). The adopted control conditions were BG-11m medium (3% $NaNO_3$, 45 mg.L^{-1}), pH adjusted to 9.5, and temperature set at 25 °C. These specific conditions were selected to closely resemble the natural conditions of the sampling site from which the material was collected.

The metric (n = 200) characterization of material from nature and culture was done under a light microscope (Zeiss Axioplan 2), considering the main morphometric characters: width and length of cells. The growth rate was calculated for the exponential phase using the formula proposed by Fogg & Thake (1987): $\mu = [\ln(N_1) - \ln(N_0) / t_1\text{-}t_0]$, where N is the number of cells at time 1 and time zero. The biovolume was calculated according to (Hillebrand et al., 1999). The Cell Yield (R) was calculated based on the cell density at the end of the experiment (30th day) minus the cell density of the initial inoculum (~1.5 × 10^5 cells.mL^{-1}) (Santos et al., 2011). For *A. elenkinii*, the ratio of heterocytes to vegetative cells was calculated for each treatment. All treatments were analyzed using analysis of variance (ANOVA) and the Tukey test, using GraphPad Prism version 5.0. Results of all tests were considered significant at a 95% confidence level if p<0.05.

3. Results

3.1. Occurrence of L. platensis and A. elenkinii in Pantanal da Nhecolândia

L. platensis occurred in the saline-alkaline lagoons of the Pantanal in pH values ranging from 9.22 to 10.23 (mean 9.9), electrical conductivity ranging from 716 to 19,020 µS.cm^{-1}, and temperatures ranging from 22.8 to 34.1 °C (average 29.0 °C) (Figure 1; Table 1). In the Salitrada Campo Dora lagoon, the species was recorded only once, during the dry-to-wet transition period, with a pH of 8.4, electrical conductivity of 1,852 µS.cm^{-1}, and temperature of 32 °C. These data, derived from 19 samples, highlight the specificity of alkaline pH, high conductivity, and warm temperatures for the occurrence of *L. platensis* in the Pantanal lagoons.

A. elenkinii was found in all water samples collected from the salt lagoons, exhibiting abundance during both dry and flood periods. For this species, the pH values ranged from 9.04 to 10.4 (average 9.8), electrical conductivity ranged from 716 to 19,020 µS.cm^{-1} (average 6,413), and temperature ranged from 21.7 to 36.1 °C (average 29 °C) (Figure 1; Table 1). The species primarily formed blooms during dry periods in the Salina do Meio, Salina Pedra do Sol, Salina da Ponta, and Salina da Reserva lagoons. In the Salitrada Campo Dora and Baía da Sede Nhumirim lagoons, *A. elenkinii* was found in only one sample, poorly abundant, with pH values of 8.4 and 7.8, electrical conductivity of 1,852 and 2,020 µS.cm^{-1}, and temperatures of 32 °C and 35 °C, respectively (Table 1).

3.2. Ecophysiological studies on L. platensis and A. elenkinii

3.2.1. Ecophysiological studies on *L. platensis* CCIBt3335

In our experiments, the relationship between growth and temperature (25, 30, and 35 °C) could not be confirmed, as there was no growth of the species at 30 or 35 °C (Tables 2 and 3). The experiments with *L. platensis* CCIBt3335 confirmed that the strain requires a high concentration of nitrogen or a highly alkaline medium for its growth. Treatments with 3% or 0% nitrate at pH 7 or 9.5 (C, T2, T3, T4, T6) were inhibitory to the species' growth (Table 2 and Figure 2). In T5, with a pH of 10.5, the species was able to grow even in a nitrogen-limited culture medium (3% nitrate), indicating its adaptability to an alkaline environment. In the condition with higher

Table 2. Experiments control and treatment conditions.

	pH	Temp. °C	Nitrate mg.L^{-1} (BG11 modified)
Control - C	9.5	25	45 (3% $NaNO_3$ of BG11 original)
Treatment 1 – T1	9.5	25	**750 (50%** $NaNO_3$ of BG11 original**)**
Treatment 2 – T2	9.5	25	**0 (0%** $NaNO_3$ of BG11 original**)**
Treatment 3 – T3	9.5	**30**	45
Treatment 4 – T4	9.5	**35**	45
Treatment 5 – T5	**10.5**	25	45
Treatment 6 – T6	**7.0**	25	45

nitrogen availability (T1- 50% nitrate, pH 9.5), the strain exhibited the highest cell yield (30 days) and growth rate (in exponential phase), significantly differing from T5 (3% nitrate, pH 10.5) (p<0.05) (Table 3, Figures 2 and 3).

3.2.2. Ecophysiological studies on *A. elenkinii* CCIBt1059

Figure 4 shows the growth curves for all treatments of *A. elenkinii*, while Figure 5 presents the growth curves and the heterocyte/vegetative cell density ratio (=heterocyte frequency) for experiments comparing nitrate concentration (T1 vs T2) and temperature (T3 vs T4). The data from the pH experiment (T5 vs T6) are from Santos et al. (2011) and they are included in the discussion for comparison.

3.2.2.1. Effects of nitrogen availability (Sodium Nitrate - $NaNO_3$) on the development of *A. elenkinii* (T1 vs T2)

Table 4 and Figure 6 present the calculated growth rates (day 10) for the exponential phase

Table 3. The exponential growth phase, growth rate, and cell yield of *Limnospira platensis* CCIBt3335 under different treatments (BG-11m, photoperiod of 12-12 hours light-dark, light intensity of 80-100 µmol photons.m^{-2}.s^{-1}).

Treatments	Exponential growth phase (days)	Growth rate (10 days) (µ)	Cell yield (30 days) (cells.mL^{-1})
Control: 3% $NaNO_3$, pH 9.5, 25 °C	NO	NO	NO
T.1: 50% $NaNO_3$	9-15	0.33	5,519,083
T.2: 0% $NaNO_3$	NO	NO	NO
T.3: 30 °C	NO	NO	NO
T.4: 35 °C	NO	NO	NO
T.5: pH 10.5	9-15	0.27	2,352,417
T.6: pH 7.0	NO	NO	NO

NO = no growth observed.

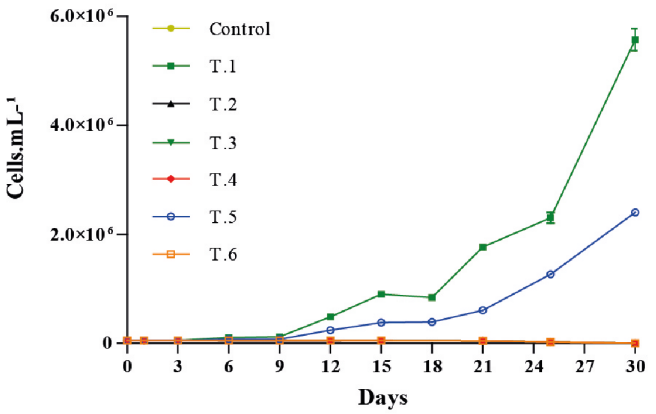


Figure 2. Growth curves of *Limnospira platensis* CCIBt3335 under different treatments. Bars indicate standard deviation.

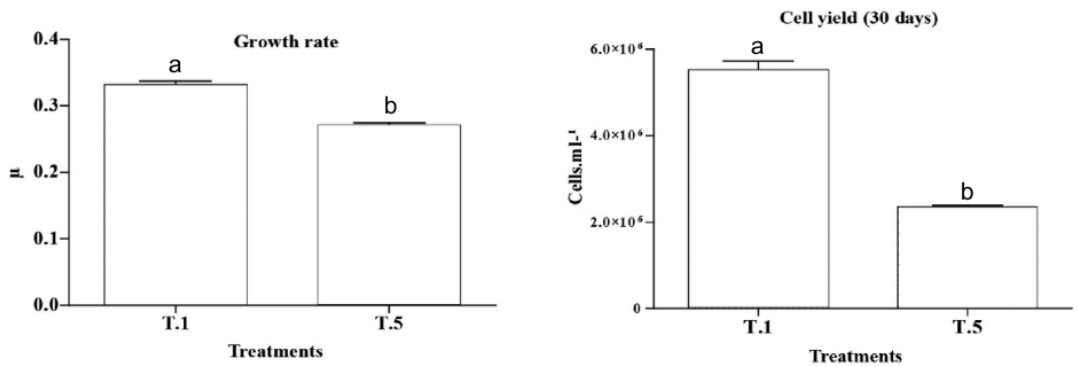


Figure 3. Growth rate and cell yield of *Limnospira platensis* CCIBt3335 under different treatments. Bars indicate standard deviation. Distinct letters indicate significative differences (p<0.05).

Table 4. Growth rate and cellular yield of *Anabaenopsos elenkinii* CCIBt1059 in different treatments. BG-11m, photoperiod of 12-12 hours light-dark, light intensity of 80-100 µmol photon.$m^{-2}.s^{-1}$.

Treatments	Exponential phase (days)	Growth rate (10 dias) (µ)	Cell yield (30 days) (cells.ml^{-1})
Control (3%$NaNO_3$, pH 9.5, 25 °C)	3-10	0.11^{a}	$800,833^{a}$
T.1 (50% $NaNO_3$)	3-14	0.19^{bd}	$9,265,000^{b}$
T.2 (0% $NaNO_3$)	7-10	0.31^{c}	$4,549,166^{c}$
T.3 (30 °C)	3-10	0.15^{abd}	$431,250^{d}$
T.4 (35 °C)	8-10	0.13^{ab}	$46,666^{e}$
*T.5 (pH 10.5)	4-12	0.21^{d}	$6,210,833^{f}$
*T.6 (pH 7.0)	6-10	0.17^{abd}	$839,166^{a}$

Means followed by different letters differ significantly from each other ($p < 0.05$). *Data of pH experiments from Santos et al. (2011).

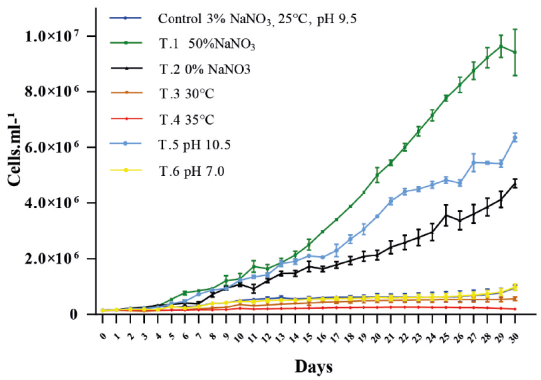


Figure 4. Growth curves of *Anabaenopsis elenkinii* CCIBt1059 under different treatments. Bars indicate standard deviation. Data of pH experiments from Santos et al. (2011).

of each treatment, as well as the cellular yield obtained at the end of the experiments (day 30). The treatment without a nitrogen source (T2) exhibited the highest growth rate in the exponential growth phase, statistically different (p<0.05) from the control (3% $NaNO_3$), and the treatment with the highest nitrogen availability in the medium (T1). The remarkable growth rate of *A. elenkinii* CCIBt1059 in the absence of nitrogen (T2) can be attributed to the increased frequency of heterocytes (Figure 5A and B).

The frequency of these specialized cells, responsible for atmospheric nitrogen fixation, increased from 15% at the beginning of the experiment to 33% on the seventh day of cultivation in T2 (Figure 5A and B). This higher frequency likely contributed to the availability of nitrogen for the vegetative cells, thereby promoting exponential growth from the seventh to the tenth day (Table 4, Figure 5A and B). However, during the exponential growth phase, the frequency of heterocytes decreased from 33% (7th day) to 27% (10th day), potentially resulting in a nitrogen deficit and a subsequent reduction and subsequent reduction in cell multiplication, culminating at the end of exponential growth (from the 10th to the 11th day - Figure 5A and B) in T2. This shift in the frequency of heterocytes from 27% (10th day) to 34% (11th day - Figure 5A and B) indicates a dynamic balance between the nitrogen demand required for cell multiplication and the capacity of heterocyte differentiation to meet this demand in a nitrogen-limited environment. The same decreasing trend in heterocyte frequency during exponential growth was observed in the control condition (Figure 5A and B).

In the treatment with higher nitrogen availability (T1), the frequency of heterocytes during the exponential growth phase was similar to that observed in the treatment without nitrogen and higher than that observed in the control condition (Figure 5A and B). However, starting from the 15th day, the frequency of heterocytes in T1 was significantly lower (p<0.05) than in T2, and from the 19th day, it was even lower than in the control condition. This clearly demonstrates that nitrogen availability in the medium and growth phases influenced the frequency of heterocytes in *A. elenkinii*. Therefore, we can establish the following relationship: the higher the nitrogen availability, the lower the frequency of heterocytes (as observed from the 15th day of cultivation), and conversely, the lower the nitrogen availability, the higher the frequency of heterocytes from the early days of cultivation (Figure 5A and B).

3.2.2.2. Effects of temperature on the growth of *A. elenkinii* (T3 vs T4)

Regarding temperature, although the growth rates (exponential phase) did not show statistical differences among the control treatment (25 °C), T3 (30 °C), and T4 (35 °C), the cellular yield was

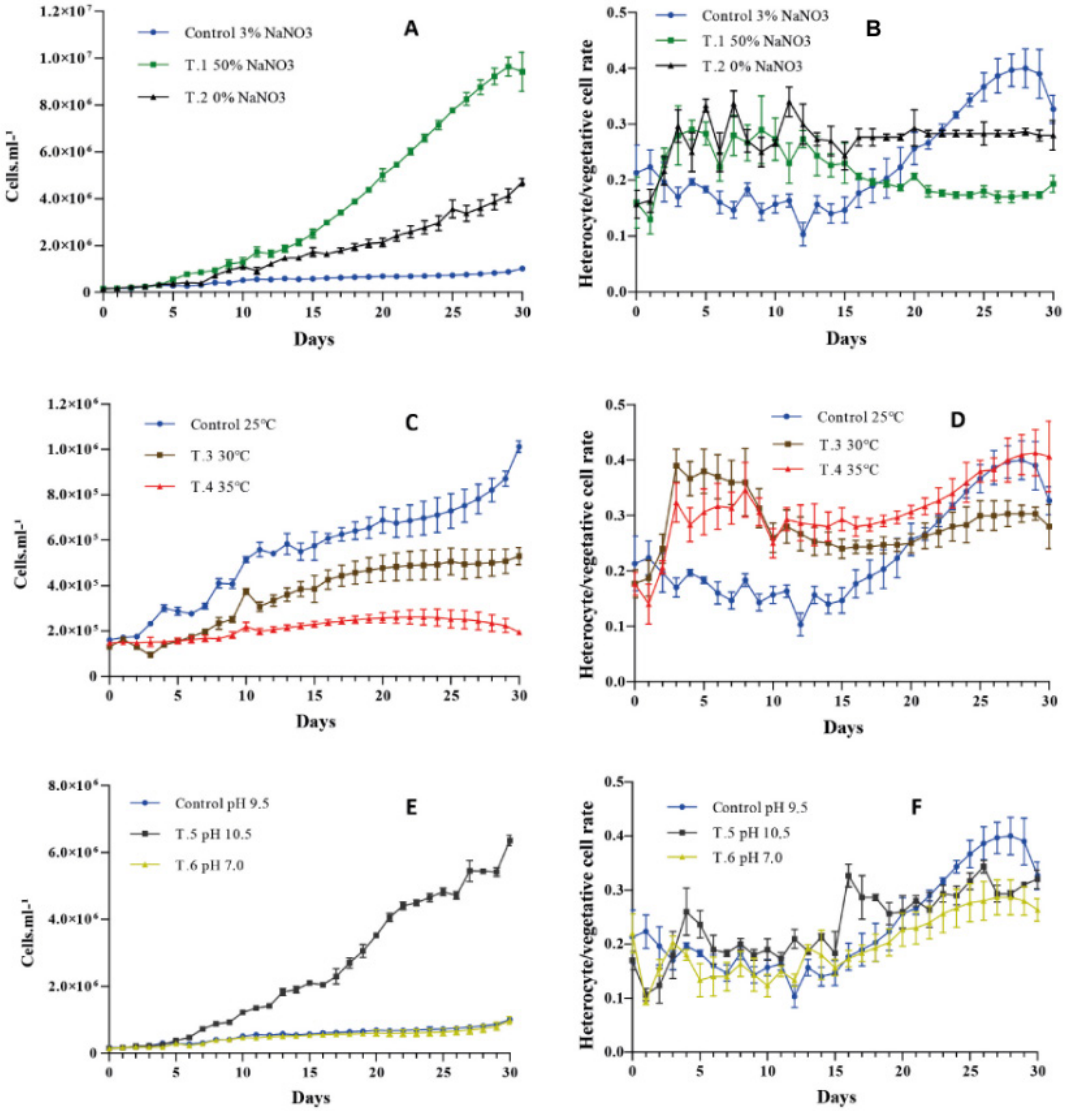


Figure 5. Growth curves (left) and heterocyte/vegetative cell ratio (right) of *Anabaenopsis elenkinii* CCIBt1059 under different treatments. (A) and (B) Nitrate experiments; (C) and (D) Temperature experiments; (E) and (F) pH experiments. Bars indicate standard deviation.

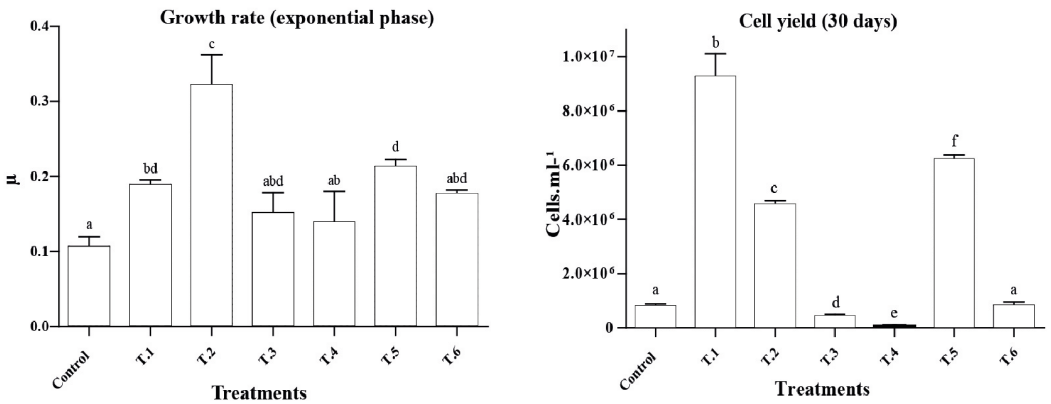


Figure 6. Growth rate (exponential phase) and cell yield (30th day) of *Anabaenopsis elenkinii* CCIBt1059 under different treatments. Bars indicate standard deviation. Distinct letters indicate significative differences (p<0.05). Data of pH experiments from Santos et al. (2011).

clearly distinct among the different temperatures tested (Table 4, Figure 5). The highest cellular yield was observed at a temperature of 25 °C (C), which was approximately 2 times higher than that observed at 30 °C (T3) and approximately 17 times higher than that observed at 35 °C (T4) (Table 4, Figure 5). These results indicate that the optimal temperature for the growth of *A. elenkinii* is 25 °C, and temperatures of 30 °C and 35 °C limit its growth.

The frequency of heterocytes during the exponential growth phase (up to the 10th day) at temperatures of 30 °C and 35 °C was statistically similar. Both temperature treatments showed a higher proportion of heterocytes compared to the control condition (25 °C) (Figure 5C and D). These results indicate that the increase in temperature influenced heterocyte differentiation, and the formation of these specialized nitrogen-fixing cells may contribute to a physiological mechanism that signals an increased demand for nitrogen to alleviate temperature stress (considering that elevated temperatures reduced the growth of the species - Figure 5C).

3.2.2.3. Effects of pH on the development of *A. elenkinii* (T5 vs T6)

The effects of different pH values - 7, 9.5, and 10.5 - corresponding to treatments T.6, control, and T.5, respectively, on the development of *A. elenkinii* CCIBt1059 were published by us in 2011 (Santos et al., 2011) and are part of the results obtained in this study.

In their work, the authors provided the first experimental evidence of the effect of pH on the development and morphology of *A. elenkinii* and demonstrated the high dependence on highly alkaline pH (10.5) for optimal species development. They showed that at lower pH values (7 and 9.5), growth limitation can occur in terms of cell density and biomass (Table 4). Regarding morphological aspects, Santos et al. (2011) demonstrated a wide variation in the length and width of vegetative cells and heterocytes, but this variation still falls within an acceptable range for *A. elenkinii* (sensu Komárek, 2005).

4. Discussion

The results showed that the low occurrence of *A. elenkinii* in freshwater lakes from Pantanal can be attributed to the fact that salitrada lagoons and bays typically contain freshwater with pH levels around neutral (Mourão et al., 1988; Mourão, 1989; Santos & Sant'Anna 2010), which are not suitable conditions for this species. However, during extended drought periods, these lagoons may experience increased nutrient concentrations and higher pH values (Almeida et al., 2011), creating favorable conditions for the higher occurrence of the species.

These data, derived from 27 samples, highlight the specificity of alkaline pH, high conductivity, and warm temperatures for the occurrence of *A. elenkinii* in the Pantanal lagoons (Table 1).

According to this, the occasional dominance of *L. platensis* (non-heterocytous) in the 19 samples Nhecolândia salines may indicate high pH values and/or greater nitrogen availability in these lagoons, considering that the occurrence of the species was limited to pH values of 9.2-10.2, electrical conductivity of 716-19,020 $\mu S.cm^{-1}$, and temperatures of 22.8-33.3 °C (Table 1).

Similar results were obtained in detailed studies conducted by Iltis (1968, 1969), where a correlation was found between the biomass density of *Limnospira* and the high salinity (conductivity) of alkaline lakes in East Africa (Chad). Massive blooms of *Limnospira fusiformis* (cited as *Arthrospira fusiformis*) were found at salinity values of 22-62 $g.L^{-1}$ (ups), pH 8.5-11.0, and temperatures of 25-40 °C (Iltis, 1968; Vonshak & Tomaselli, 2000).

As cited above, our experimental results have demonstrated that under highly alkaline pH conditions (pH 10.5), the growth of *L. platensis* can occur even at low nitrate concentrations (BG-11 3%, $NaNO_3$ 45 $mg.L^{-1}$). It should be noted that the strain did not exhibit any growth at pH 7.0 and 9.5, even with the same available nitrogen concentration. However, the highest growth rate and cell yield were observed in the condition with the highest nitrate availability (T1- BG-11 50%, $NaNO_3$ 750 $mg.L^{-1}$) at pH 9.5. Also, there was no growth observed in the absence of a nitrogen source (BG-11 0%) or at 3% nitrate concentration at pH 9.5. This result indicates that both nitrogen availability and high pH influence the growth of *L. platensis*.

This is aligned with Mourão (1989), which states that the high pH values and low Nitrogen/Phosphorus ratio that occur in the Salina do Meio lagoon are likely the primary limiting factors for the growth of many algae and cyanobacteria species. This is because only 1% of the nitrogen present in this lagoon is available in a form that can be assimilated by the phytoplankton (Mourão, 1989). As a result, only species that are adapted or have

strategies to fix other forms of nitrogen would be capable of thriving in this extreme environment.

Considering this, to explain the presence of blooms in the studied lagoons of the Pantanal, the available nitrogen may have originated from leaching of the soil through rainwater or from the feces of migratory birds, cattle, and wildlife that frequent the saltwater lagoons. In addition to these factors, the decomposition of the biomass from *A. elenkinii* blooms (a nitrogen-fixing heterocytous cyanobacterium that forms frequent blooms in the salines) provides the necessary nitrogen for the growth and subsequent blooming of *L. platensis* in the Pantanal salt lagoons. However, the replacement of dominance between *L. platensis* and *A. elenkinii* in the Pantanal salt lagoons is not yet fully understood, and only specific studies with weekly or daily *in situ* monitoring could help explain this intriguing process.

In fact, the increased conductivity levels in the environment promotes the formation of blooms of *L. platensis*, which is also commonly found in Lake Naivasha and Lake Oloiden in Kenya (Ballot et al., 2009 - cited as *Arthrospira platensis*). These lakes serve as typical examples of recent degradation and salinization, occurring within the past years, due to excessive human water abstraction and neglect in managing eutrophication. The blooms observed in these lakes are a direct response to the significant deterioration of the natural environmental conditions and the progressive rise in nutrient concentrations. The transformation of Lake Oloiden, originally a freshwater lake, into a hypereutrophic and alkaline state, has resulted in a shift in species dominance from coccoid Chlorophyceae to the prevalence of *Limnospira fusiformis* (cited as *Arthrospira fusiformis*) and *A. elenkinii*. The primary sources of nutrient input into this lake are livestock herds and the detergents used for laundry washing within the lake. These findings highlight the threat posed to the domestic use of water from Lake Oloiden due to its inadequate physicochemical conditions (Ballot et al., 2009).

In the Brazilian Pantanal, Malone et al. (2012) demonstrated that the effects of natural and/or anthropogenic alterations, which allow the influx of freshwater into salt lagoons, disrupt the typical flora of these environments. The authors further emphasized the importance of preserving the natural isolation characteristics of the salt lagoons and highlighted that the composition of their microflora is a valuable tool for assessing ecological changes in these systems.

The increase in heterocytes frequency in response to nitrogen concentration reduction has also been documented in several cyanobacteria species studied by Zapomelová et al., (2008), including *Anabaena planctonica* Brunnthaler, *Anabaena sphaerica* f. *conoidea* Elenkin, *Anabaena spiroides* Klebahn, *Aphanizomenon gracile* (Lemmermann) Lemmermann, *Nostoc* sp., *Scytonema* sp., and *Tolypothrix* sp. Similar findings were reported for *Anabaena planctonica* Brunnthaler by Wood et al. (2010), *Anabaena cylindrica* Lemmermann by Adams et al. (1981), and *Cylindrospermopsis raciborskii* by Padisák (1997), among others. However, for *A. elenkinii*, the data from this study are novel and provide new insights into its heterocytes formation.

In the saline lagoons of the Pantanal, nitrogen deficiency is considered the limiting factor for phytoplankton growth (Medina-Júnior & Rietzler, 2005). Therefore, it is expected that heterocytous cyanobacteria dominate in these systems, as is the case with the dominance of *A. elenkinii* in these alkaline lagoons (Santos & Sant'Anna, 2010). However, our experiments results show that under conditions of abundant nitrogen in the medium (T1), this species can proliferate more extensively than under nitrogen-deficient conditions after 30 days in culture (Table 4, Figure 5A).

From an ecophysiological perspective, it is likely that these growth responses of *A. elenkinii* to extreme conditions of high nitrogen availability (T.1) or complete nitrogen deprivation (T.2) also occur in natural conditions. According to Mourão (1989) and Medina-Júnior & Rietzler (2005), the total nitrogen concentration in the Salina do Meio lagoon during the dry season is 2 to 3 times higher than during the rainy season.

During the period sampled in this study, Santos (2008) reported nutrient data for the Salina do Meio lagoon in the dry season (28/VIII/2006), which included Total Phosphorus (TP) 13,357 μg L^{-1}, Orthophosphate 3,496 μg L^{-1}, Total Nitrogen (TN) 6,437 μg $L^{-}1$, Nitrate 3,999 μg L^{-1}, Nitrite 393 μg L^{-1}, and Ammonia 21,016 μg L^{-1}; and in the rainy season (04/V/2007): TP 1,943 μg L^{-1}, Orthophosphate 23.5 μg L^{-1}, TN 11,660 μg L^{-1}, Nitrate 17.7 μg L^{-1}, Nitrite 6.7 μg L^{-1}, and Ammonia 1,097 μg L^{-1}. Although TN is approximately 2 times higher in the rainy season than in the dry season, the high concentration of ammonia in the dry season (19 times higher than in the rainy season) helps to explain the predominance of cyanobacteria in this lagoon.

As reported by Santos & Sant'Anna (2010) and supported by our present study, blooms of *A. elenkinii* are mainly observed in this lagoon during the dry periods. This highlights the species' adaptive capacity to the seasonal variations that occur in the saline lagoons of the Pantanal, justifying its dominance in both dry and rainy periods.

Dolman et al. (2012), performed a study on 102 lakes in the north of Germany, and found *A. elenkinii* dominating in lakes with low nitrogen and high phosphorus concentrations. Probably, these conditions are similar to those of Pantanal Salinas. However, Dolman et al. (2012) did not consider pH and conductivity data in their study, which made comparison difficult.

In terms of morphometric analysis, we selected the width of vegetative cells and heterocytes (the specialized cells to fix nitrogen) to illustrate the effect of the treatments on these features. As observed in Figure 7A and B, there is a clear tendency for an increase in cell width during the exponential growth phase and a significant reduction during the stationary phase. Compared to the natural material (Salina do Meio, collected on 19/august/2009), the width of vegetative cells was significantly larger during the exponential growth phase in the control, T1, and T2 treatments (Figure 7A and B), and during the stationary phase, there was a reduction in cell width in all treatments, approaching the values observed in the natural sample. Despite the wide variation in cells and heterocytes width under culture conditions, this variability remains within the documented range for the species (approximately 4-6 µm cell width and 3-5 µm heterocyte width) and is considered part of the morphological variability of *A. elenkinii* (Komárek, 2005) - (Figure 7).

Considering that our results show a negative effect of increasing temperature on the growth of *A. elenkinii*, probable global warming of about 1.4 to 5.8 °C over the next 100 years could lead to a reduction in the growth of *A. elenkinii* in the saline lagoons of the Pantanal and even allow for the replacement of species. This fact could bring important modifications to the biological and biogeochemical processes of these tropical alkaline lagoons and could also favor the dispersal of *A. elenkinii* to regions with milder temperatures, such as countries in temperate regions (Wiedner et al., 2007).

The correlation between heterocyte frequency and temperature has been reported for the first time by Zapomelová et al. (2008), for

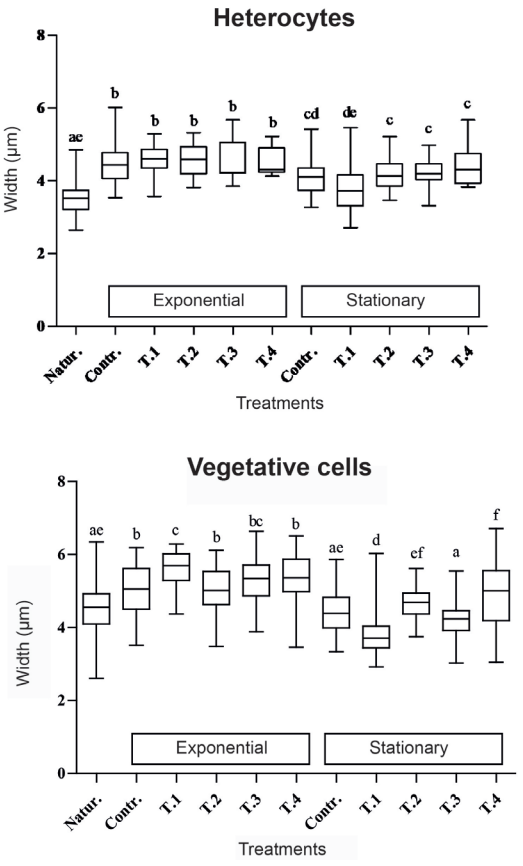


Figure 7. Width of vegetative cells (A) and heterocytes (B) of *Anabaenopsis elenkinii* CCIBT1059 on nature (Salina do Meio) and under different treatments, in the exponential and stationary growth phases. Whiskers represent minimum and maximum values, boxes symbolize ± standard deviation, and "-" inside boxes are mean values. Distinct letters indicate significant differences (p<0.05).

species of *Dolichospermum* (cited as planktonic *Anabaena*), *Tolypothrix* sp. and *Aphanizomenon gracile* Lemmermann. Our results confirm these findings for *A. elenkinii*.

The effects of temperature have not been studied for other isolates of *A. elenkinii*, which makes it difficult to compare with our results. However, several studies have been conducted with other cyanobacteria species. For example, Saker and Griffiths (2000) studied the effect of temperature (ranging from 20 to 35 °C) on the growth of seven strains of *Raphidiopsis raciborskii* (Woloszynska) Aguilera & al. (cited as *Cylindrospermopsis raciborskii*) isolated from northern Australia, and the highest growth rates for all strains were observed between 25 and 30 °C. Castro et al. (2004) investigated the effect of temperatures of 19 and 25 °C on the growth of a strain of the same species isolated from the Billings Reservoir (São Paulo, Brazil) and

documented three times greater growth at 25 °C compared to cultivation at 19 °C. Moreover, a study by Bittencourt-Oliveira & Molica (2003) with spiral and straight strains of *C. raciborskii*, isolated from Brazil at temperatures of 21 and 31 °C, showed that both morphotypes exhibited higher growth rates at 31 °C.

Santos et al. (2011) did not find akinetes in any of the pH treatments studied and argued that pH is unlikely to influence the differentiation of this resting cell in *A. elenkinii*. However, the effect of pH did influence akinete germination in the species *A. arnoldii* Aptekar (Reddy 1984), indicating that the response to this parameter varies depending on the species.

Similar to the pH treatments (Santos et al., 2011), no akinetes were observed in any of the other tested treatments. These results suggest that akinete formation in *A. elenkinii* does not seem to be linked to changes in pH, nitrogen availability, and temperature, at least within the tested range of variations.

Considering that akinetes are formed under stressful conditions or when cultures approach the stationary phase (Moore et al., 2005), we conducted two pilot experiments in an attempt to induce stress conditions in *A. elenkinii* CCIBt1059. In the first experiment, we exposed the strain to continuous light (80-100 µmol photons.m^2.s^{-1}) for 60 days. In the second experiment, we kept the strain in darkness for 40 days. In both conditions (continuous light and darkness), we maintained the strain in a BG-11 medium with 3% nitrogen, at a temperature of 25 °C, and a pH of 10.5 (according to T5). We made observations every three days. The strain did not produce akinetes under either of these tested conditions. Thus, the question 'which factors influence the formation of akinetes in *A. elenkinii*?' remains unanswered.

It is worth noting that in samples from the saline lagoons of the Pantanal, trichomes with akinetes are common and occur in both blooming samples (especially during dry periods) and samples with lower dominance of this species (during flood periods). In natural conditions, there must be some signaling factor for akinete formation, which appears to be related to competition among other phytoplankton species, particularly the dominant diatom, *Craticula guaykuruorum* C.E.Wetzel, E.A.Morales & Ector (Santos et al., 2012 – cited as *Craticula* cf. *buderi* (Hustedt) Lange-Bertalot), and the non-heterocyteous cyanobacterium *L. platensis*. However, further studies are needed to address these hypotheses.

5. Conclusion

We have found that the treatment with the highest nitrogen availability (T1: BG-11 medium with 50% NaNO3, 750 mg.L^{-1}), pH of 9.5, a temperature of 25 °C, a photoperiod of 12-12 hours of light-dark, and light intensity between 80-100 µmol photons m^{-2} s^{-1} provides the most favorable conditions for the growth of both studied species. We recommend the use of these conditions for future studies, for example, to evaluate the protein and lipid concentrations of these strains and their potential use in the food industry. Additionally, the *L. platensis* strain also exhibited growth in treatment 5, which had a pH of 10.5 and BG-11 medium with 3% $NaNO_3$ (45 mg.L^{-1}) along with other identical conditions compared to other treatments. This finding highlights the species' strong dependence on both high nitrogen availability and high pH.

The growth of *A. elenkinii* is positively correlated with an increase in pH, and the optimal condition for its growth is the pH calibrated at 10.5, underscoring the alkaliphilic nature of this species.

Furthermore, we observed that temperature influences the frequency of heterocyte formation in *A. elenkinii*. This study is one of the few in the literature to show that temperature, in addition to nitrogen availability, affects the frequency of these nitrogen-fixing specialized cells in cyanobacteria. At elevated temperatures (30 and 35 °C), the frequency of heterocytes was higher compared to 25 °C during the exponential growth phase, indicating that increased heterocyte formation is a strategy in response to temperature stress.

This research provides valuable insights into the ecological aspects and optimization of the cultivation of the two species studied, which hold ecological significance for saline lakes. Further studies are recommended to explore their potential biotechnological applications, for example, for human and animal consumption.

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