



Effect of different nutrition media on the growth of blue-green algae (*Microcystis* sp.) isolated from striped catfish (*Pangasianodon hypophthalmus*) farming ponds

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Abstract. Blue-green algae can multiply quickly in nutrient-rich environments. The study was carried out to determine the appropriate medium and nutrient content for the growth of *Microcystis* sp. The study consisted of two experiments conducted in an indoor hatchery. *Microcystis* sp. algae was cultured in 8-liter plastic bottles with a light intensity of 3,000 Lux using white LED light and an aeration time of 24/24 hours. In experiment 1, *Microcystis* sp. was separately cultured in different mediums, namely BG11, Zarrouk, and NPK (16:16:8). Each treatment was repeated three times. The results indicated that the density of *Microcystis* sp. was the highest in the NPK treatment (16.2×10^6 cells mL⁻¹). In addition, dry weight (58.02 g L⁻¹) and chlorophyll-a content (4,054 µg L⁻¹) in the NPK treatment had higher densities than other mediums after 8 days of culture. For the experiment 2, when using NPK cultured at doses of 50, 75, 100, and 150 mg L⁻¹, the results showed that the algal abundance was the highest (14.5×10^6 cells mL⁻¹) in the 50 mg L⁻¹ dose after 10 days of culture. In summary, inorganic fertilizer NPK at a concentration of 50 mg L⁻¹ can be used effectively in the mass cultivation of *Microcystis* sp. microalgae.

Key Words: dry weight, chlorophyll-a, harmful algae, NPK, water quality.

Introduction. Blue-green algae, also known as cyanobacteria, are considered a group of toxic algae with the risk of reducing productivity and yield in aquaculture (Lee & Jones 1991). However, their acute or chronic effects on mussels (reduced ability to eat), small crustaceans, and arthropods (Landsberg 2002), cause pernicious toxicity to freshwater fish such as seabream, common carp, eel, perch, milkfish, climbing perch, and trout (Rodger et al 1994; Landsberg 2002). Among them, the blue-green algae *Microcystis aeruginosa* is the most common species that predominates in nutrient-rich water environments with strong light intensity and high temperatures in freshwater bodies. When overgrown, it forms large algae clumps, secreting microcystin (MC) toxins that can cause a direct threat to fish, crustaceans, and mollusks (Aguilera et al 2018; Beck & Wu 2021). Additionally, the potential environmental and health risks caused by *Microcystis* blooms and their toxins make them an ecologically and economically important species (Mohan et al 2023).

Cyanobacteria are well recognized for their ability to fix atmospheric nitrogen. This group holds significant value as a major natural food source for cultural forms. But a significant number of them are also known for their nuisance due to their ability to produce potentially lethal toxins (Kumar & Sinha 2014). According to Guo (2007), blue-green algal blooms caused by lake eutrophication have posed severe risks to the environment in China. The blue-green algal blooms in Taihu Lake, Chaohu Lake, and Tianchi Lake are mainly attributable to the overwhelming proliferation and aggregation of *Microcystis aeruginosa*, which generally occurs in large colonies with high algal cell densities in eutrophic lakes (Welker et al 2004). Phosphorus has long been seen as playing a major role in primary production, and as such, it is widely regarded as the limiting nutrient in freshwater ecosystems. Like other primary producers, *M. aeruginosa*

feeds on nutrients that are either introduced to or cycled throughout the water, and, with the help of sunlight, rapidly blooms in dense colonies under eutrophic conditions. Not all strains of *M. aeruginosa* are toxic, nor are all strains colony-forming (Ma et al 2014). Nitrogen (N) and phosphorus (P) over-enrichment accelerates eutrophication and promoted cyanobacterial blooms worldwide. The colonial bloom-forming cyanobacterial genus *Microcystis* is covered by sheaths, which can protect cells from zooplankton grazing, viral or bacterial attack, and other potential negative environmental factors (Ma et al 2014). Nitrogen addition to lake water maintained *Microcystis* colony size, promoted growth of total phytoplankton, and increased *Microcystis* proportion as part of total phytoplankton biomass. Increases in phosphorus did not promote growth but led to smaller colonies, and had no significant impact on the proportion of *Microcystis* in the community. N and P addition together promoted phytoplankton growth much more than only adding N (Ma et al 2014). Therefore, the objective of this study is to assess the influence of various nutrient media on the development of *Microcystis* sp. isolated from catfish culture ponds. The findings will contribute to managing water quality and limiting the harmful algae bloom in the fresh water aquaculture area.

Material and Method

Study time and location. The study was conducted from January-May 2023 at the Laboratory of Live Food, Faculty of Aquatic Biology and Environment, College of Aquaculture and Fisheries, Can Tho University.

Experiment design. Algae *Microcystis* sp. were isolated (Figure 1 and Figure 2) from striped catfish ponds in O Mon district, Can Tho city. Materials used in this study include twelve plastic jars with a volume of 8 L, an electromagnetic scale with 2 odd numbers, a multiparameter portable to measure temperature and pH, an LED light, etc. The media for algae culture were BG11 medium (Stanier et al 1971), Zarrouk medium (Zarrouk 1966), and NPK (16:16:8). Algal culture water was treated with Javel and continuous aeration for 24 hours before conducting experiments.

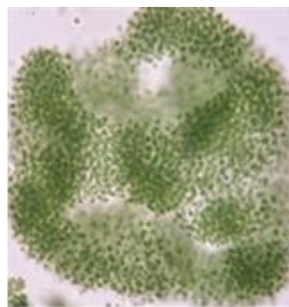


Figure 1. Colony of *Microcystis* sp.

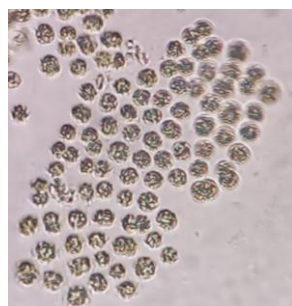


Figure 2. Cells of *Microcystis* sp. after separation.

Experimental condition. The experiment was conducted in a laboratory and the algae *Microcystis* sp. was raised in plastic jars with a volume of 8 L (the experimental water volume was 7 L). The initial algae density was 1×10^6 cells mL^{-1} . Light was provided by

LED lights, white light with a light intensity of 3,000 Lux, 24/7 lighting, and continuous aeration.

Experiment 1: The influence of nutrient media on the growth of *Microcystis* sp.

The experiment used three types of nutrient media consisting of BG11, Zarouk, and NPK (16:16:8) to culture the algae of *Microcystis* sp. A completely randomized design was used, with 3 treatments and 3 replicates for each treatment. The dosages of BG11, Zarrouk, and NPK media were 10, 100, and 1 mL L⁻¹ added in algae culture water treatments, respectively. The experiment (Figure 3a) was performed over a period of 10 days.

Experiment 2: Effect of NPK concentration on the development of the algae *Microcystis* sp.

Algae *Microcystis* sp. was cultured with four various levels of NPK, including 50 (T50), 75 (T75), 100 (T100), and 150 mg L⁻¹ (T150). The experimental conditions were similar to the first experiment. The NPK medium was prepared by dissolving 50 g of NPK in 1 L of distilled water and autoclaving it. At this time, 1 mL of solution was equivalent to 0.05 g of NPK. Then, NPK medium was added to the experimental jars at predetermined concentrations. The experiment (Figure 3b) time was 13 days.



Figure 3a. Experiment 1.



Figure 3b. Experiment 2.

Sampling parameters. Temperature and pH were measured twice a day at 8:00 AM and 2:00 PM using a multiparameter portable. Nutrient contents, including total ammonium nitrogen (TAN), nitrate (NO₃⁻), and phosphate (PO₄³⁻) were sampled every 3 days (100 mL each time). Samples were stored at 4±1°C and analyzed according to APHA (2017). The algae of *Microcystis* sp. was collected daily with a volume of 10 mL, stored in glass bottles, and fixed with formalin at a concentration of 4%. Then, 2-3 drops of lugol were added to the algal samples, shaken and stored for 1-2 days for cells in the colony to separate. The algae were counted by a Burkner counting chamber. Algal density was estimated according to Coutteau (1996):

$$\text{Algal density (cell mL}^{-1}\text{)} = \frac{n_1 + n_2}{160} \times 10^6 \times d$$

Where:

n₁ - cell number of the first chamber (cell mL⁻¹);
 n₂ - cell number of the second chamber (cell mL⁻¹);
 d - dilution factor.

Identify the density of algae.

Dry weight of algae. The algal samples were collected on day 6 with a volume of 5 mL each filtered through a Whatman 0.22 µm filter paper (dried at 60°C for 2 hours). Then, the algal samples were dried in an oven at a temperature of 60°C for 24 hours. The dry weight of the algae was measured by an electronic scale (4 odd numbers).

Concentration of chlorophyll-a. 50 mL of algal samples were collected every 3 days. The concentration of Chlorophyll-a was measured by spectrophotometer at wavelengths of 630, 647, 664, and 750 nm. The algal density was calculated according to the following formula (APHA 2017):

$$\text{Chlorophyll-a (µg/L)} = [11.85(E_{664} - E_{750}) - 1.54(E_{647} - E_{750}) - 0.08(E_{630} - E_{750})] \times [(V_1 \times 1.000)] / V_2$$

Where:

V₁ - volume of acetone (50 mL);
 V₂ - volume of filtered water.

Size of algae. Algal cells were measured randomly (at least 100 algal cells) using an eyepiece micrometer in 3 periods, including the beginning of the experiment, the end of the rapid growth period (day 6), and the end of the experiment.

Data analysis. The data was calculated as the mean and standard deviation. Differences between treatments were analyzed by Statistica 8.0 software using the Duncan test at a statistical significance level of p<0.05.

Results

The influence of nutrient media on the growth of *Microcystis* sp.

Water quality parameters. The temperature during the sampling periods fluctuated low, and there was no statistically significant difference (p>0.05) between the three treatments in the morning (28.1-28.3°C), while a statistically significant difference (p<0.05) was found between the Zarrouk and the NPK treatments (32.5–32.9°C) in the afternoon. The pH values ranged from 7.9 to 9.0 and showed a significant difference between the NPK treatment and the remaining treatments both in the morning and afternoon (Table 1).

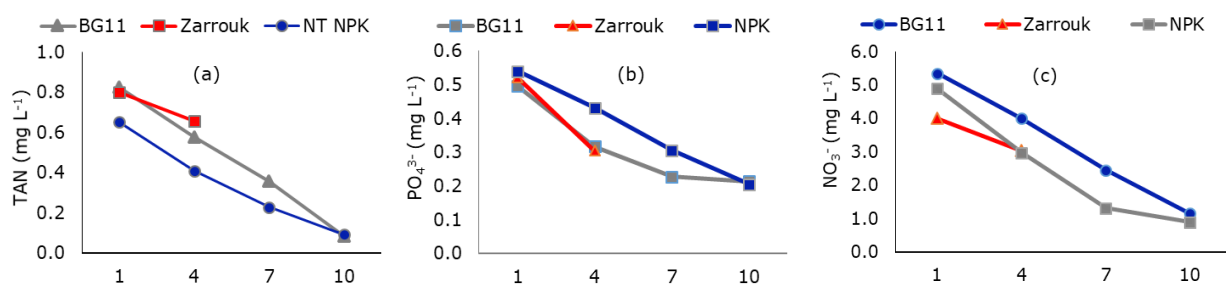
Table 1

Mean temperature (°C) and pH in experiment 1

No.	Treatment	Temperature (°C)		pH	
		Morning	Afternoon	Morning	Afternoon
1	BG11	28.2±0.1 ^a	32.7±0.1 ^{ab}	9.0±0.4 ^b	8.6±0.4 ^b
2	Zarrouk	28.1±0.2 ^a	32.5±0.1 ^a	9.0±0.1 ^b	9.0±0.2 ^b
3	NPK	28.3±0.2 ^a	32.9±0.2 ^b	8.2±0.5 ^a	7.9±0.2 ^a

The values with different superscript letters in a column are significantly different ($p < 0.05$).

After one day of culture, the TAN, NO_3^- , and PO_4^{3-} contents (Figure 4) in the treatments ranged from 0.652-0.825 mg L^{-1} , 4.005-5.353 mg L^{-1} , 0.496-0.540 mg L^{-1} , respectively. The levels of TAN, NO_3^- , and PO_4^{3-} decreased gradually through the sampling stages. These contents showed a statistically significant difference ($p < 0.05$) between treatments across sampling periods.

Figure 4. Concentrations of (a) TAN, (b) PO_4^{3-} , and NO_3^- in experiment 1.

The growth of algae in different nutrition media. The *Microcystis* sp. abundance reached the highest level in the NPK treatment on day 8 (16.2×10^6 cells mL^{-1}) and was significantly different ($p < 0.05$) from the other treatments. Treatment BG11 had the second highest density on day 8 (9.8×10^6 cells mL^{-1}) and treatment Zarrouk revealed the lowest density (2.3×10^6 cells mL^{-1}) on day 3 of culture cycle (Figure 5).

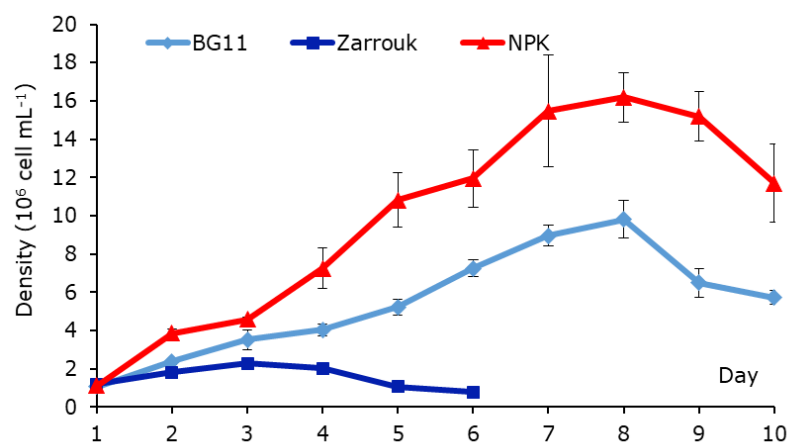


Figure 5. Algal density of treatments during the sampling periods in experiment 1.

Algal size. The initial average size of *Microcystis* sp. had a length of 4.81 ± 1.65 μm . After 10 days of culture, the size of *Microcystis* sp. in BG11, Zarrouk, and NPK treatments was 5.20 ± 1.61 μm , 5.15 ± 1.59 μm , 5.26 ± 1.58 μm , respectively (Table 2). The algae size in all treatments increased towards the end of the experiment, but there was no significant difference among treatments.

Table 2

Average size of *Microcystis* sp. in experiment 1

Treatment	Declining relative growth phase (day 6)	End of experiment (day 10)
BG11	5.13±1.65 ^a	5.20±1.61 ^a
Zarrouk	5.09±1.64 ^a	5.15±1.59 ^a
NPK	5.15±1.59 ^a	5.26±1.58 ^a

The values with different superscript letters in a column are significantly different ($p < 0.05$).

Dry weight. Dry weight of *Microcystis* sp. changed from 11.58–58.02 g L⁻¹ on day 6 of the culture cycle. The algal weight showed the highest level in the NPK treatment (58.02±0.05 g L⁻¹), followed by the BG11 treatment (35.55±0.08 g L⁻¹). The dry weight of algae was the lowest in the Zarrouk treatment (11.58±0.03 g L⁻¹) and had a statistically significant difference ($p < 0.05$) compared to the other treatments (Figure 6).

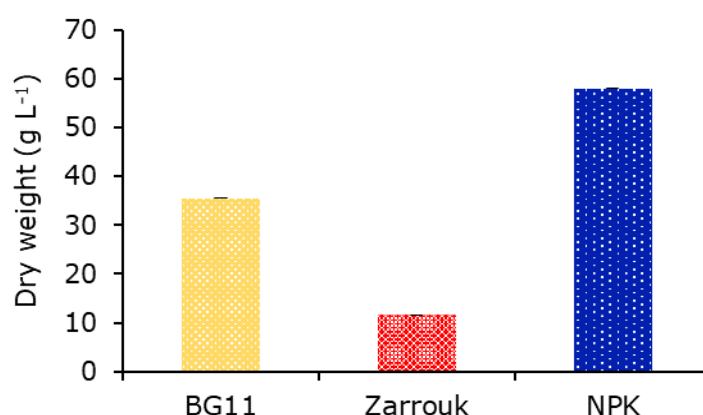


Figure 6. Dry weight of algae in experiment 1.

Chlorophyll-a. The chlorophyll-a contents reached its highest level in the NPK treatment (4,054±0.96 µg L⁻¹) on day 7. Meanwhile, chlorophyll-a level in the Zarrouk treatment had the lowest concentration (1,025±0.94 µg L⁻¹) and algae only grew until day 4. The algal population declined earlier than other treatments (Table 3).

Table 3

Contents of chlorophyll-a (µg L⁻¹) in experiment 1

Treatments	Day 1	Day 4	Day 7	Day 10
BG11	821±0.96 ^a	1,825±0.96 ^b	2,520±0.08 ^a	1,541±0.61 ^a
Zarrouk	850±0.88 ^b	1,026±0.94 ^a	-	-
NPK	856±0.94 ^c	2,331±0.94 ^c	4,054±0.96 ^b	2,277±0.84 ^b

The values with different superscript letters in a column are significantly different ($p < 0.05$).

Experiment 2. Effect of NPK concentration on the growth of *Microcystis* sp.

Water environment parameters. The temperature among treatments in the experiment had a low variation and was not statistically significant ($p \geq 0.05$) across the sampling phases. Temperatures measured in the morning and afternoon were 28.6–28.7°C and 32.3–32.6°C, respectively. The average pH of the treatments changed from 7.9–8.7. A significant difference was found between the pH value in the 50 mg L⁻¹ treatment in the afternoon ($p < 0.05$) compared to the other treatments (Table 4).

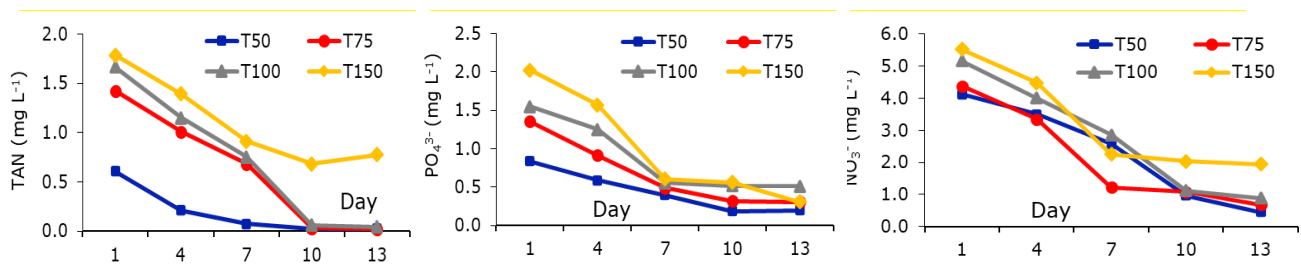
Table 4

Mean temperature (°C) and pH values in experiment 2

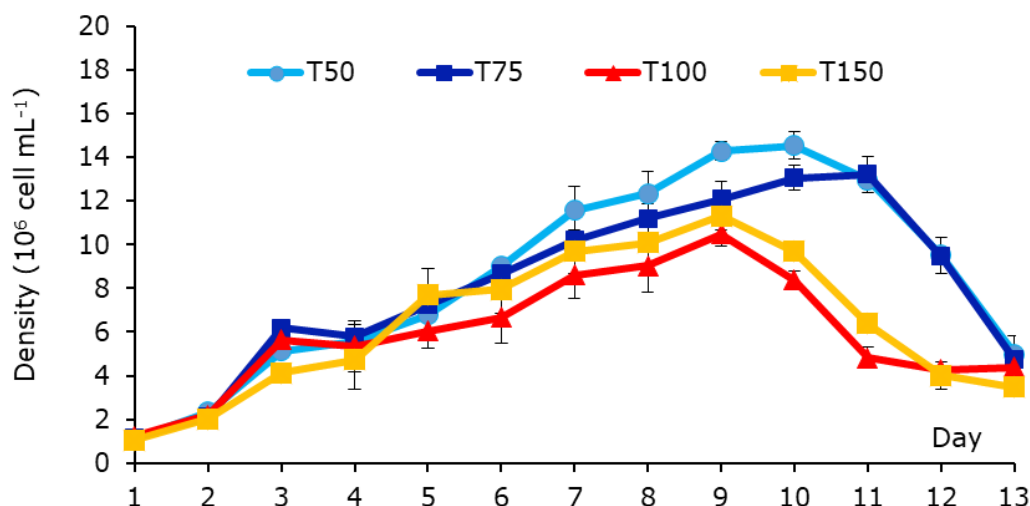
No.	Treatment	Temperature (°C)		pH	
		Morning	Afternoon	Morning	Afternoon
1	T50	28.6±0.1 ^a	32.6±0.1 ^a	8.7±0.3 ^b	8.7±0.3 ^b
2	T75	28.6±0.1 ^a	32.5±0.2 ^a	7.9±0.2 ^a	8.2±0.2 ^a
3	T100	28.6±0.4 ^a	32.3±0.2 ^a	7.9±0.2 ^a	8.1±0.2 ^a
4	T150	28.7±0.1 ^a	32.5±0.1 ^a	7.7±0.2 ^a	7.9±0.2 ^a

The values with different superscript letters in a column are significantly different ($p < 0.05$).

The TAN, NO_3^- and PO_4^{3-} contents (Figure 7) in all treatments decreased during study periods. Levels of nutrition were significantly different ($p < 0.05$) between treatments. TAN, NO_3^- and PO_4^{3-} concentrations varied from 0.609–1.787 mg L^{-1} , 4.130–5.531 mg L^{-1} , 0.838–2.025 mg L^{-1} , respectively.

Figure 7. Concentrations of TAN, NO_3^- , and PO_4^{3-} in experiment 2.

Variation in algal density during the sampling stages. The results from Figure 8 showed that in the treatment of NPK at a dosage of 50 mg L^{-1} , the algal density reached its maximum on day 10 (14.5×10^6 cells mL^{-1}) and the difference was statistically significant ($p < 0.05$) compared to the remaining treatments. Algal density on day 11 in all treatments tended to gradually decrease according to the culture cycle.

Figure 8. Abundance of *Microcystis* sp. in experiment 2.

Algal size. The average size of *Microcystis* sp. was 5.15 ± 1.41 μm on the first day of culture. After 13 days of culture, the average sizes in the 50, 75, 100 and 150 mg L^{-1} treatments were 5.24 ± 1.55 μm , 5.20 ± 1.69 μm , 5.21 ± 1.64 μm , and 5.20 ± 1.55 μm , respectively. By the end of the experiment (day 13), algae size in all treatments

increased, but the difference was not statistically significant ($p > 0.05$) among treatments (Table 5).

Table 5

Mean size of *Microcystis* sp. in experiment 2

Treatment	Declining relative growth phase (Day 6)	End of experiment (Day 13)
Initial	5.15±1.41 ^a	5.15±1.41 ^a
T50	5.12±1.62 ^a	5.24±1.55 ^a
T75	5.07±1.68 ^a	5.20±1.69 ^a
T100	5.14±1.62 ^a	5.21±1.64 ^a
T150	5.13±1.46 ^a	5.20±1.55 ^a

The values with different superscript letters in a column are significantly different ($p < 0.05$).

Dry weight of algae. The average dry weight of *Microcystis* sp. ranged from 36.63-43.46 g L⁻¹ (Figure 9). The algal weight difference in the 50 mg L⁻¹ treatment was significantly different ($p < 0.05$) compared to the 3 treatments of 75, 100, and 150 mg L⁻¹. The highest weight was obtained in the 50 mg L⁻¹ treatment and the lowest in the 100 mg L⁻¹ treatment.

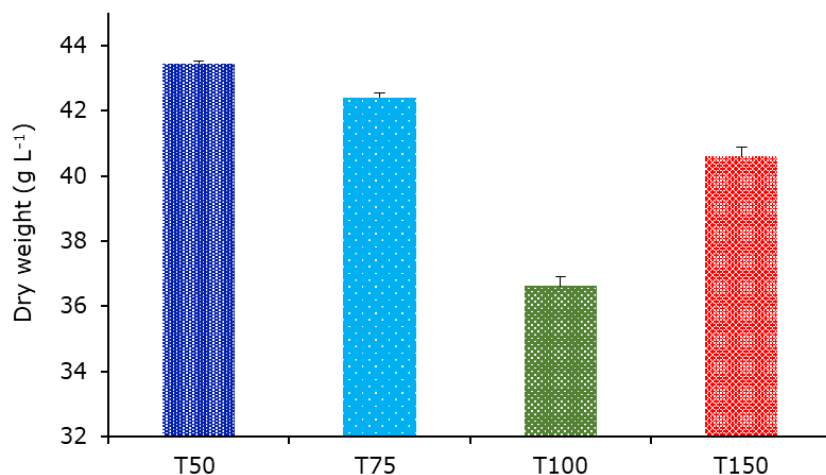


Figure 9. Dry weight of *Microcystis* sp. in experiment 2.

Content of chlorophyll-a. Chlorophyll-a content tended to increase with culture time, reaching its highest value on day 10. Then, it decreased in all treatments on day 13. Chlorophyll-a level was the highest in the 50 mg L⁻¹ treatment (4,028±1.0 mg L⁻¹) and the lowest in the 100 mg L⁻¹ treatment (1,538±0.5 µg L⁻¹) (Table 6).

Table 6

Concentration of chlorophyll-a (µg L⁻¹) in experiment 2

No.	Treatment	Day 1	Day 4	Day 7	Day 10	Day 13
1	T50	843±1.6 ^d	1,3340±0.9 ^c	3,309±0.9 ^d	4,028±1.0 ^d	1,314±0.6 ^d
2	T75	824±1.0 ^b	1,395±0.9 ^d	3,067±0.9 ^c	3,259±0.9 ^c	890±1.6 ^c
2	T100	830±0.9 ^c	1,165±0.8 ^b	1,104±0.9 ^a	1,538±0.5 ^a	564±0.2 ^b
3	T150	805±0.1 ^a	993±0.9 ^a	1,501±0.8 ^b	2,201±1.0 ^b	329±1.0 ^a

The values with different superscript letters in a column are significantly different ($p < 0.05$).

Discussion. Temperatures ranging from 18.2-32.5°C were suitable for the growth of *Microcystis* algae (Chen et al 2003). In addition, the water temperature of the screened ponds ranges from 24.8-31.0°C (Kumar & Sinha 2014). This temperature range was

optimal for the growth of cyanobacteria in aquatic systems (Howard & Easthope 2002). Blue-green algae grew optimally with a pH range of 7.5-9.0 (Wei et al 2001) or from 7.5-10 (Fogg 1956). Research by Hoda et al (2012) showed that two species, *Microcystis aeruginosa*, and *M. flos-aquae* grew well when the pH changed from 7-11. Nutrients, including TAN, NO_3^- and PO_4^{3-} , tended to gradually decrease with algal development. Algae absorbed nutrients for growth, the contents in the treatments decreased at the end of the end of the experiment. According to Te & Gin (2011), *Microcystis* sp. density seemed to be positively related to total nitrogen and total phosphorus concentrations. Algae required less phosphorus when compared with nitrogen. However, phosphorus was an essential factor for algal growth. Yang et al (2018) found that when the pH ranged from 7-8, *M. aeruginosa* reached a maximum density of 10.35×10^4 cells mL^{-1} after 19 days of culture at a volume of 250 mL in BG11 medium at a temperature of 25°C, with a lighting time of 14 light hours and 10 dark hours. This revealed that the algae density in that study was lower than the findings of the current study. Consequently, it can be seen that culture conditions affect the growth of algae. Different cultivation conditions of microalgae may affect microalgal growth rates, biomass yield and their nutritional content in terms of lipid and fatty acid production (Zhu et al 2014; Zienkiewicz et al 2020). Additionally, the cell density of *M. aeruginosa* bloom from an aquaculture pond in Gazipur, Bangladesh, was 6.22×10^8 cells L^{-1} . During the bloom, dissolved oxygen and nitrite nitrogen in pond water were recorded at 4.5 and 0.68 mg L^{-1} , respectively (Ahmed et al 2008).

The results from Table 2 showed that *Microcystis* sp. culture in NPK medium had the highest cell size. The algal size in the treatments was consistent with the observations of Gibson et al (1990), *Microcystis* sp. cell size varied between 2-7 μm . The dry mass of algae in experiment 1 had a positive correlation with the algal density. The abundance of *Microcystis* sp. was the lowest in the Zarrouk medium. Chlorophyll-a content was relatively high and tended to fluctuate proportionally with changes in algal density. According to Trung et al (2018), high cyanobacteria chlorophyll-a concentrations from 1,437 to 5,100 $\mu\text{g L}^{-1}$ were measured in several carp ponds in Ho Chi Minh City, in a duck-fish pond and in extensive catfish ponds in Southern Vietnam. The abundance of cyanobacteria in terms of chlorophyll-a was strongly correlated with TN ($r = 0.709$) and TP ($r = 0.676$). From the findings of this experiment, it can be concluded that NPK can be used well in *Microcystis* sp. cultivation with a dosage of 50 mg L^{-1} . The algal density identified a maximum of 16.2×10^6 cells mL^{-1} after 10 days of culture in NPK medium. In addition, dry weight and chlorophyll-a contents were 58.02 ± 0.05 g L^{-1} and $4,054 \pm 0.96$ $\mu\text{g L}^{-1}$, respectively. These values were much higher than those found in Zarrouk and BG11 media. According to Smith (1983), blue-green algae are the dominant phytoplankton in lentic systems when the TN:TP ratio is <29 , and nitrate is the main nutrient content for algal growth (Richmond 1986). However, the TAN element at the end of the experiment in the treatment using NPK (150 mg L^{-1}) tended to increase again. This was because the algae density at the end of the experiment decreased and the decomposition of the algae caused the TAN content to increase.

The higher the NPK content, the lower the algae density achieved in this study (Figure 8). These findings were similar to the result of Rofidi (2017): a commercial fertilizer (NPK fertilizer) was used to grow algae of *Chlorella* sp. with concentrations of 70, 700, and 1,330 mg L^{-1} , respectively. A maximum algal density was recorded at the 70 mg L^{-1} treatment. Similarly, Hien et al (2021) applied three types of Walne, BG11, and NPK media to cultivate the *Scenedesmus* sp. algae biomass. The results revealed that the algal growth was the best in the NPK treatment with a level of 50 mg L^{-1} . Various nitrogen and phosphorus concentrations in microalgae cultivation medium may influence the lipid and fatty acid yield (Yang et al 2008; Xin et al 2010). When increasing NPK content, the accumulation of nutritional components inside the cells of *Microcystis* sp. also increased, and therefore algal cell size also rose, but no significant difference ($p > 0.05$) was found between treatments. In the experiment, Thuy et al (2017) reported that the size of *Microcystis* algae cells is about 3-7 μm . Chlorophyll-a concentration in each culture decreased as the amount of nitrogen or phosphorus supplied to the medium was reduced. At a lower nitrate concentration, a significantly higher concentration of

microcystin was measured ($p < 0.001$), showing a direct relationship between nutrient limitation and an increase in microcystin (Pimentel & Giani 2014). However, chlorophyll-a content in this study tended to decrease in treatments supplemented with higher levels of NPK. Nitrogen (N), phosphorus (P), and/or their ratio may limit the floating plants' growth. Both N and P can be limiting or co-limiting in aquatic systems (Elser et al 2007). In general, the density and biomass of *Microcystis* sp. algae reached the highest level (14.5×10^6 cells mL^{-1}) when using NPK with a dosage of 50 mg L^{-1} after 10 days of culture. In addition, the NPK 150 mg L^{-1} treatment also had a relatively high algal density on day 9 and a decrease on day 10. However, the maximum algal density of this treatment was lower (11.3×10^6 cells mL^{-1}) compared to the treatment using NPK 50 mg L^{-1} .

Conclusions. Medium of NPK with a content of 50 mg L^{-1} was suitable for the growth of *Microcystis* sp. The average algae density reached a maximum of $14.5\text{-}16.2 \times 10^6$ cells mL^{-1} after 8-10 days of cultivation. In addition, dry weight and chlorophyll-a levels also reached their highest levels when culturing *Microcystis* sp. with NPK. Further research is needed on the *Microcystis* sp. algal biomass culture with different N:P ratios. In addition, *Microcystis* sp. combined cultivation with some freshwater fish should also be done in order to evaluate the effects of these potentially harmful algae on their growth.

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Conflict of interest. The authors declare no conflict of interest.

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