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The Effect of *Azolla microphylla* Liquid Fertilizer on the Growth of *Nannochloropsis oculata* Populations on a Laboratory Scale

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ABSTRACT

Nannochloropsis oculata is a microalgae often used as natural food for fish larvae. In general, microalgae cultivation uses standardized Walne fertilizer. This research uses an experimental method to determine the effect of administering *Azolla microphylla* liquid fertilizer at different doses on the growth of the *N. oculata* population on a laboratory scale. This experiment was conducted from September to October 2022 at the Experimental Pond Laboratory, Biotechnology Laboratory, Marine Biology Laboratory, Department of Marine Science, Faculty of Fisheries and Marine, Universitas Riau. This research used a Simple, Completely Randomized Design (CRD) with four treatment levels and three replications. The treatment used *A. microphylla* liquid fertilizer with doses A (2 mL), B (3 mL), C (4 mL), and D (Walne fertilizer control). The results showed that the highest population density was in treatment D (control) on day 6 with a value of 483.5 x 10^5 (cells/mL) and treatment C (4 mL *A.microphylla* fertilizer) on day 5 with a value of 437.3 x 10^5 (cells/mL). The results of the ANOVA test showed a significance value of 0.007 < 0.05, which indicated a significant difference between the treatment of *A. microphylla* liquid organic fertilizer at different doses on the density of *N.oculata*. Relatively, the growth rate of *N. oculata* in treatment D (Walne fertilizer) was still the highest, with a value of 170.868%, and treatment C, with a percentage of 145.005%. Furthermore, the specific growth rate in treatment B was the highest, with a value of 1.730% on day 4. The Walne liquid fertilizer treatment, which was the control, was still higher and dominated the population and growth rate of *N. oculata* and could not be replaced with *A. microphylla* liquid fertilizer.

Keywords: *Azolla*, Microalgae, Walne.

1. INTRODUCTION

Feed in cultivation consists of natural and artificial feed. Even though the development of artificial feed is increasing rapidly, the use of artificial feed still cannot completely replace the function of natural feed. Natural food has high nutritional content, improves organic matter composition, and maintains water quality. Raw food is often used when hatching larvae.

One natural food often used for cultivating larvae is *Nannochloropsis oculata*. According to Meria et al. (2021); Sagala et al. (2022), *N. oculata* is a green microalgae, round in shape and small in size, with a 2-4 micrometers diameter. Laboratory and mass-scale *N. oculata* cultures generally use standardized PA (pro-analysis) inorganic fertilizers such as Walne fertilizer as a source of nutrient production. This dependence causes the need to develop alternative fertilizers to increase the *N. oculata* population to maintain stocks for breeding purposes. One alternative organic fertilizer for cultivating microalgae is water weeds such as *Azolla microphylla* (Isnansetyo and Kurniastuty, 1995).

The use of *A. microphylla* as a liquid fertilizer in microalgae cultivation was chosen because it has a high nutrient content (Etikawati and Jutono, 2000), so it is very suitable for the growth of *N. oculata*. Knowing the effective dose needed to achieve maximum growth is just necessary. The absence of research regarding the use of *A. microphylla* liquid fertilizer on the growth of *N.oculata* makes the author interested in researching this matter. This research aims to determine the effect of providing *A. microphylla* liquid fertilizer with different concentrations on the growth of the *N. oculata* population.

2. RESEARCH METHOD

Time and Place

This research was carried out from

September to October 2022 at the Experimental Pond Laboratory, Biotechnology Laboratory, and Marine Biology Laboratory, Department of Marine Science, Faculty of Fisheries and Marine, Universitas Riau. This research began with making *A. microphylla* liquid fertilizer, preparing cultivation media, cultivation, and counting. Population density of *N. oculata* and examination of water quality of cultivation media.

Method

This research used experimental methods and a Completely Randomized Design (CRD) with four treatment levels and three replications, according to Tanjung (2014). The treatment of the study consisted of *A.microphylla* liquid fertilizer with doses A (2 mL), B (3 mL), C (4 mL), and D (Walne fertilizer control) on the growth of the *N.oculata* population.

Procedures

Fertilizer Preparation

This research used Walne liquid fertilizer obtained from online purchases. Meanwhile, *A.microphylla* was obtained from a research pond at the Reservoir, Faculty of Fisheries and Marine, Universitas Riau. Making A. microphylla liquid fertilizer follows the procedure (Albab, 2017; Putra et al., 2022): 1) Wash *A.microphylla* thoroughly, then dry it in the hot sun for three days. 2) Grind *A.microphylla* into flour. 3) Dissolve *A.microphylla* flour in akuades in a ratio of 1:4 (500 g *A.microphylla* dissolved in 2 L of akuades) for 3-4 weeks. 4) Squeeze and strain the soaking to get *A.microphylla* liquid fertilizer. 5) Sterilize *A.microphylla* liquid fertilizer using an autoclave. 6) Store liquid fertilizer in a closed, sterile glass container to avoid contamination and fermentation until ready to use

Next, Proximate analysis was carried out on 500 ml of *A. microphylla* liquid organic fertilizer, which had been prepared and fermented first as supporting data for the research.

Cultural Media

Culture media was made by mixing 8400 ml of seawater (33 ppt) with 2800 mL of freshwater (0 ppt) in a ratio of 3:1 (3 seawater + 1 freshwater). So, the salinity with the best value is obtained at 25 ppt. The prepared media was put into 700 mL/jars, each into 12 treatment jars.

Fertilization

Fertilization was done by adding A. microphylla liquid fertilizer to treatments A1, A2, A3, B1, B2, B3, C1, C2, C3 and Walne fertilizer to treatments D1, D2 and D3. Fertilization is carried out first before the seeds are sown so that the nutrients contained therein can be distributed evenly and absorbed optimally for the growth of *N.oculata*.

Seed Dispersal

Seed distribution was carried out by pouring *N.oculata* seeds into each culture container with a ratio of 7:3 (700 mL of culture media and 300 ml of *N. oculata* seeds). The *N.oculata* seeds used had an initial density of 178.5 x 10^5 cells/mL.

Aeration and Maintenance

Setting and maintaining aeration by using an aerator as an oxygen producer is one of the limiting factors in cultivating *N. oculata*. Cultivation is carried out for seven days while maintaining and checking the cultivation medium's temperature, salinity, and pH to ensure they remain in the best condition.

Measured Parameters

Population Growth of *N. oculata*

Sampel *N. oculata* was taken 1 mL in culture medium and diluted with a mixture of 9 ml of distilled water. Then, take one sample drop, put it in the hemocytometer, and cover it with a cover slip. Then, observe under a microscope with 100 times magnification. Calculations were carried out using a hand counter with three repetitions to minimize errors. Analysis of the density of *N. oculata* in the hemocytometer box uses the formula according to Ahmadi et al. (2019) as follows: The formula for Calculating density:

$$P = \frac{n \; x \; 25 \; x \; 10000}{5}$$

Description:

P = Population density of *N. oculata* (cells/mL)

n = Number of hemocytometer observations

25 = Number of hemocytometer counts

5 = The number of hemocytometer sections was counted.

Relative Growth Rate (RGR)= $\frac{Ct-Co}{Co}$ 100%

Description:

RGR = Relative Growth Rate (%)
Co = Cell population density at the beginning of observation (cells/mL)
Ct = Cell population density at the end of observation (cells/mL)

$$SGR = ((Ct/co)^{1/t} - 1) \times 100\%$$

Description:

SGR = Spesific Growth Rate (%)
Co = Cell population density at the beginning of observation (cells/mL)
Ct = Cell population density at the end of observation (cells/mL)
t = Length of observation period (hours)

Measurement of Culture Media Water Quality

Water quality measurements were carried out every day for seven consecutive days at 10.00 WIB in all experimental jars. Light intensity and dissolved oxygen were not measured and were considered constant. Water quality measurements include the cultivation media's temperature, salinity, and pH.

Data Analysis

The primary data from the research is presented in tables and graphs and then discussed using descriptive statistics. To test the effect of giving *A. microphylla* liquid fertilizer at different doses on the growth of the *N. oculata* population, Analysis of Variance (ANOVA) was carried out with a significance level of 5% (0.05) on the IMB SPSS Statistics software on the peak day of *N. oculata* growth. The Least Significant Difference (LSD) further test was carried out to compare each treatment.

3. RESULT AND DISCUSSION

Population Growth *N.oculata*

The growth of the *N. oculata* population in the culture process was characterized by an increase in the number of cells over time of observation. *N. oculata* cell growth is divided into 5 phases: the resting phase, exponential phase, decreasing growth rate phase, stationary phase, and death phase. The results of observations of the average population density of *N. oculata* can be seen in Table 1.

Table 1. Average cell density of *N. oculata* (cells/mL)

Days to-	Density (cells/mL) x 10^5 (x 10^5)			
	A	B	C	D
1	178,5 ± 0,00	178,5 ± 0,00	178,5 ± 0,00	178,5 ± 0,00
2	165,3 ± 12,4	172,8 ± 2,60	187,3 ± 5,60	193,8 ± 7,80
3	241,0 ± 5,30	285,8 ± 25,6	301,0 ± 16,6	261,5 ± 25,9
4	330,3 ± 20,3	406,7 ± 11,3	324,0 ± 26,2	389,2 ± 8,00
5	322,0 ± 33,4	398,2 ± 11,5	437,3 ± 18,7	396,5 ± 17,5
6	128,5 ± 66,9	230,5 ± 60,7	291,5 ± 40,4	483,5 ± 14,9
7	84,4 ± 60,8	141,5 ± 32,2	211,3 ± 23,2	254,2 ± 31,4

The highest density of *N. oculata* was obtained on day 6 of observation in the Walne liquid fertilizer treatment with a value of 483.5x10^5 cells/mL. The highest density in the *A.microphylla* liquid fertilizer treatment was treatment C on day 5 with a value of 43.7 x 10^5 cells/ml, treatment B on day 4 with 406.7 x 10^5 cells/ml, and treatment A on day 4 with a value of 330.3 x 10^5 cells/ml.

The day after culture (day 2), *N.oculata* underwent several adjustments (adaptation phase). This is characterized by a decrease in the Number of cells that lasts 24 hours in treatments A and B. The population does not experience significant changes. The organisms undergo metabolism, but cell division has not occurred, so cell density does not increase. However, this did not happen in treatments (C) and D (control). The population of *N. oculata* is greatly influenced by the nutrient levels in the cultivation medium, which are necessary for its growth and survival.

Each treatment experienced an exponential phase after day 2 and lasted until close to day 4 for treatments A and B. Meanwhile, this phase took place for treatment C and reached peak growth on day 5. In contrast to treatment D (control), the exponential phase was still ongoing and peaked on day 6. Differences in the length of the exponential phase could occur due to differences in the response of *N. oculata* to

each treatment dose after the initial dose. Cultivation phase until reaching peak growth. According to Mukhlis et al. (2017), the exponential phase is characterized by an increase in the density of the cell population and its growth rate by one-fold or more from the initial density until it reaches the peak population.

Treatments A and B stationary phase began from day 4 to day 5. Meanwhile, treatments C and D (control) did not experience a dormant stage but immediately experienced a death phase after the peak growth phase. The stationary phase occurs because the available nutrients can no longer help cell growth. In this phase, microalgae compete for space in the culture medium, and there is a significant decrease in nutrients, and no additional nutrients are added (Ru'yatin et al., 2019).

Furthermore, all *A. microphylla* liquid fertilizer treatments experienced a death phase after day 5 and treatment D (control) on day 6. The death phase occurs because the rate of cell reproduction and the fertilizer provided are no longer proportional to the rate of cell growth, resulting in a reduction in the Number of cells, which ends in cell death.

The use of *A. microphylla* liquid fertilizer at a dose of 4 ml in treatment C is a treatment that is close to the growth produced by Walne fertilizer in treatment D (Control). The use of *A. microphylla* as liquid organic fertilizer is an alternative that can be chosen because it is rich in nutrients and can make organic fertilizer more efficient and safe for the environment. The results of the proximal analysis of *A.microphylla* content can be seen in Table 2.

Table 2. Results of proximal analysis of *A.microphylla* content

Method	Nutrient	Content (%)
Walkley & Black	C	0,72
Kjeldhal	N-Total	2,08
Spectrophotometer	P_2O_5	0,11
AAS	K_2O	0,14

Relative Growth Rate *N. Oculata*

The relative growth rate of *N. oculata* cells is obtained from the percentage difference between the final density result and the initial density value and then divided by the initial density value. The results of calculating the relative growth rate values of *N.oculata* in the treatments can be seen in Table 3.

Table 3. Relative growth rate of *N. oculata* (%)

Days to-	Relative Growth Rate (RGR) (%)			
	A	B	C	D
1	0	0	0	0
2	-7,376	-3,175	4,949	8,590
3	35,014	60,131	68,628	46,499
4	85,061	127,825	81,513	118,020
5	80,392	123,063	145,005	122,969
6	-28,011	29,132	63,305	170,868
7	-52,754	-20,728	18,394	42,390

The highest relative growth rate percentage was in treatment D (control), with a value of 170.868% on day 6. Meanwhile, when giving different doses of *A. microphylla*, the highest relative growth rate values were obtained respectively in treatment C with a percentage of 145.005% on day 5, treatments B and A on day 4 with percentages of 127.825% and 85.061%. This relative growth rate is in line with the population growth of *N. oculata* in the highest exponential phase at 72-120 hours after cultivation.

The relative growth rates of treatments A and B are in the minus numbers with values of -7.376% and -3.175% on day 2. The relative growth rate of *N.oculata* can get a negative percentage if the initial density is lower than the final density, which occurs when the cells are still adapting and absorbing nutrients after being cultured. Furthermore, the relative growth rate will increase over time and peak on day 4. However, at the end of this study, the growth rates for treatments A and B returned to minus (-52.754% and -20.728%). According to Arfah et al. (2020), the difference in daily growth for each treatment is caused by the ability of the cells to absorb the nutrients contained in the culture medium.

Meanwhile, treatments C and D have begun to experience a gradual increase in the relative growth rate with a percentage of 4.949% and 8.590% at the observation interval on day 2. Furthermore, at the end of the observation, treatments C and D still showed an increase in the relative growth rate. The growth progress (although decreasing) is still higher than the initial density, with a growth rate percentage of 18.394% and 42.390%. Cell growth does not occur under these conditions; cell division is too slow, and death rates dominate.

According to Fery et al. (2020), after passing the growth peak, a decline phase will be characterized by cell division, which occurs no longer as fast as the previous phase and will decrease periodically. This growth rate value can be used as a benchmark to determine the media's supporting capacity for the growth of *N. oculata*. The faster and more significant the growth rate, the better the supporting capacity of the fertilizer media for growth in maintaining population.

Specific Growth Rate *N. oculata*

The specific growth rate (SGR) value was calculated to describe the increase in *N.oculata* per unit time. The results of calculating the specific growth rate value of *A. microphylla* liquid fertilizer on *N.oculata* population growth can be seen in Table 4.

Table 2. The specific growth rate of *N. oculata* (%)

Days to-	Specific Growth Rate (SGR) (%)			
	A	B	C	D
1	0	0	0	0
2	-0,327	-0,135	0,200	0,342
3	0,837	1,309	1,459	1,058
4	1,288	1,730	1,245	1,637
5	0,818	1,120	1,252	1,119
6	-0,460	0,244	0,506	1,043
7	-0,871	-0,207	0,138	0,291

The day after culture, the specific growth rate in treatments A and B decreased (minus percentage) due to the adaptation process of *N.oculata* to nutrient absorption. However, the specific growth rate in treatments C and D (control) increased on days 2 to 4 and decreased significantly on the following days. The highest percentage of specific growth rate in the exponential growth phase was in treatment B on day 4, with a value of 1.730% from the start of cultivation.

Meanwhile, the lowest specific growth rate was found in treatment A on day 4, with a percentage of 1.288%. Furthermore, in treatment D (control), the specific growth rate on day 4 had a percentage value of 1.637%. Different things were found in treatment C, where the highest specific growth rate in the exponential phase was found on day 3 with a percentage of 1.459%. According to Afriza et al. (2015), nutrient (fertilizer) concentrations that are too high can cause these nutrients to be too complex for cells to absorb. Using types of nutrients and doses that are too high or low can result in the absorption and growth of microalgae not being optimal.

The specific growth rate in treatment B was the highest but was not consistent. It could not replace the growth in the Walne fertilizer treatment (control), which had high nitrogen and nutrient content and was still more dominant than the other treatments. Even though the specific growth rate on Walne fertilizer media was slightly lower than treatment B, population growth continued until day 6. Meanwhile, population growth in treatments A and B only reached day 4, and treatment C peaked on day 5. The type and nutritional content of the culture media can influence the specific growth rate of *N.oculata* in the exponential phase.

Water Quality

The culture process in a closed room requires lighting in the form of lights to regulate temperature stability. The lamp used is a 40-watt LED lamp, which can produce 3000 lumens of light. The high and low temperatures are influenced by the amount of lamp power and how close the lamp is to the culture container.

The average temperature in each

N.oculata treatment ranged from 25-30°C. The temperature range of the culture media corresponds to the best range for the growth of *N. oculata*. According to Utama et al. (2015), *A. microphylla* is more tolerant of relatively high temperatures, so it is very good when cultivated in tropical climate conditions such as Indonesia. The results of measuring the temperature quality of the culture container can be seen in Figure 1.

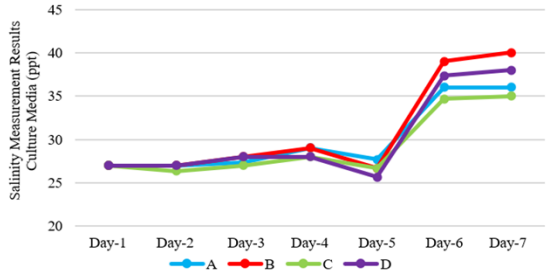

Figure 1. Culture media salinity measurement

pH Culture Media

Adding liquid organic fertilizer can affect the cultivation media's acidity (pH). Changes in pH in the algae cultivation media can occur due to the photosynthesis process, residual algae metabolism, evaporation of the water content in the cultivation media, or other methods so that the pH affects the performance of enzymes in cell metabolism Isnandina and Hermana (2013). The average pH measurement results during the study for each treatment ranged from 7.7 – 8.4 and was included in the good *N. oculata* growth range according to Wadyaningrum et al. (2013), with a pH range of 7-9.5. The results of measuring the pH of the culture media can be seen in Figure 2.

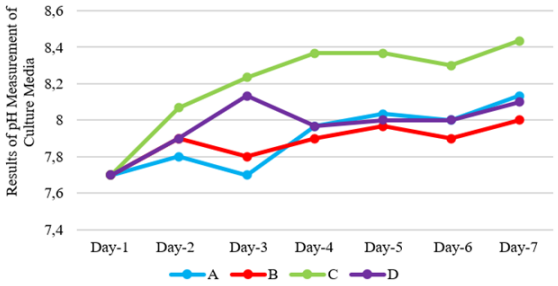

Figure 2. Culture media pH measurement

4. CONCLUSION

There was a significantly different effect of administering different doses of *A.microphylla* liquid fertilizer on the growth of the *N. oculata* population on a laboratory scale. The Walne liquid fertilizer treatment as a control was still better and dominated the population and growth rate of *N.oculata*. It could not be replaced by *A. microphylla* liquid fertilizer in this study. However, graphically and statistically, administering *A. microphylla* liquid fertilizer at a dose of 4 mL approached the population and relative growth rate of Walne's liquid fertilizer.

Further research needs to be carried out regarding the use of *A. microphylla* liquid fertilizer at higher doses or different combinations on *N. oculata* population growth so that it can be used on a large scale for the cultivation and production of *N. oculata.*

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