Growth of *Nannochloropsis oculata* in shrimp cultivation waste at difference N:P ratios

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Abstract. Shrimp culture waste contains high nutrients, namely N₂, P₂O₅, K₂O and has the potential to be used as organic fertilizer. Shrimp waste has a low N:P ratio so that it does not support microalgae growth, N:P needs to be adjusted for microalgae growth. The purpose of this study is determine the optimal N:P ratio in shrimp waste fertilizer which produced the highest growth of *Nannochloropsis oculata*. Method in this research is an experimental method. Completely Randomized Design (CRD) with 6 treatments and 3 replications. Treatment of shrimp waste fertilizer was used with different N: P ratios, N:P ratios of 10.5:1, 15.5:1, 20.5:1, and 25.5:1, namely : Walne fertilizer, N:P ratio 17:1 and Shrimp waste fertilizer with a ratio of N:P 5.9:1 is the control. Cultivation of *N. oculata* uses an initial density of 10⁵ cells/mL, with light intensity ranging from 5.010⁻⁵.622 lux for 24 hours and lasts for seven days. Data analysis was processed used ANOVA then with DMRT. The results of this study had a significantly different effect on the population and growth rate of *N. oculata*. The exponential phase highest population mean of 965x10⁴ cells/mL and the growth rate of 135x10⁴ cells/mL/day.

1. Introduction

Intensive, superintensive vaname shrimp culture uses artificial feed which has the potential as a supplier of nutrient wastes in the waters [1]. The amount of sedimentary waste produced by shrimp ponds with a density of 750 seeds/m², 1,000 seeds/m² and 1,250 seeds/m² was 18.2 tons, 20.3 tons and 21.9 tons, respectively [2]. The cultivation waste results in a decrease in the quality of the environment, that is a decrease in dissolved oxygen and an increase in ammonia levels because the effluent discharges contain toxic organic matter and nutrients, both particulate and dissolved [3]. Utilization of residual waste from shrimp farming into liquid fertilizer should be tried for use in the cultivation of natural food such as *N. oculata*. *N. oculata* has important nutrients namely Eicosapentaenoic Acid (EPA) of 30.5%, Omega 3 HUFAs at 42.7%, lipid content between 31-68% dry weight and protein content of 52.11% [4]. Nutrients that have an important role in phytoplankton growth and metabolism are N and P [5]. Limitations of elements N and P can cause microalgae cells to decrease in protein content by degradation of various cell components related to protein synthesis [6].

Shrimp culture waste is processed with the addition of Effective Microorganism (EM) to convert organic material into inorganic material in the process of making fertilizer. EM degradation process in fertilizer processing aims to ensure that the important nutrients in organic matter can be absorbed well by microalgae [7]. Hidayati [8], reported shrimp culture waste fermented with EM had an N and P
content of 181.92 mg/L and 29.42 mg/L increased to N 207.20 mg/L and P 37.11 mg/L. Ratio N: P in fermented shrimp farming waste is still low. Whereas the optimal N: P ratio for fertilizer in *N. oculata* is from 10-20: 1 [9]. The N: P ratio of shrimp aquaculture waste can be increased by enriching nutrients by adding nitrogen to shrimp aquaculture waste to stimulate the growth of *N. oculata*. N:P ratio affects the availability of nutrients in the media to carry out metabolism in the body of microalgae in the process of photosynthesis, nitrogen and phosphorus acting as a constituent of proteins in cells [6].

Shrimp farming waste which is processed into fertilizer with the addition of urea to increase the N: P ratio and fermented with Effective Microorganism (EM) as a nutrient provider in the cultivation of natural food *N. oculata* is still not found, the purpose of this study was to determine the optimal N:P ratio of shrimp waste fertilizer which can produce the highest growth in *N. oculata* cultivation.

2. Materials and methods

2.1. Research design

The method in this research was experimental methods. This research was used a completely randomized design with 6 treatments and 3 replications. This research was used the independent variable, it was the different of N:P ratio in the effluent of shrimp culture waste for the culture of *N. oculata*. The dependent variable in this research was the number of population *N. oculata* and the rate of growth of *N. oculata*. The variable control in this research was the origin of a pure culture of *N. oculata*, effective microorganism waste shrimp culture, and culture environmental conditions or water quality include light intensity, temperature, pH and salinity, pH.

The treatment of this research was the addition of the shrimp culture waste fertilizer with the same total nitrogen (N) but with different of N:P ratio. This research was used the variation of N:P ratio in 6 treatments.

**Table 1.** Treatment and concentration of cultivation waste fertilizer used for the culture of *Nannochloropsis oculata*

| Treatment | Addition of Nitrogen (mg urea/100 ml) | Nitrogen value (mg/L) | P values (mg/L) | The ratio of N:P | volume (mL/L) |
|-----------|--------------------------------------|-----------------------|-----------------|------------------|---------------|
| P0        | Walne                                | x                     | 1510            | 87.24            | 17:1          | = 1           |
| P1        | SFW                                  | x                     | 1510            | 253              | 5.9:1         | 7             |
| P2        | SFW+Urea                             | x                     | 1510            | 141              | 10.5:1        | 3.9           |
| P3        | SFW+Urea                             | 36.43 mg              | 1510            | 94               | 15.5:1        | 2.6           |
| P4        | SFW+Urea                             | 79.49 mg              | 1510            | 72               | 20.5:1        | 2.03          |
| P5        | SFW+Urea                             | 116.55 mg             | 1510            | 57               | 25.5:1        | 1.6           |

2.2. Shrimp culture waste fertilizer enriched urea

The shrimp culture waste was obtained from the intensive shrimp farming ponds in Instalasi Budidaya Air Payau (IBAP) Lamongan. The sedimentary waste of shrimp ponds was dried in the sun for 8 days. After dried shrimp farming waste, shrimp farming waste is fermented for 14 days with activated microorganisms that have been activated. Effective microorganisms contain *Saccharomyces cerevisiae* and *Lactobacillus casei*. The minimum density of *Lactobacillus casei* is $2 \times 10^6$ cells/mL and the minimum density of *S. cerevisiae* is $3.5 \times 10^5$ cells/mL [10]. *S. cerevisiae* and *L. casei* produced extracellular enzymes that break down of molecules organic matter became inorganic materials that can be easy absorbed for metabolism [11].

The activation EM was used the ratio 1:1:20 namely EM: Molasses : Aquades [12] of 1000 mL, the mixture is stirred and allowed to stand for 12 hours under anaerobic conditions [13]. The liquid fertilizer made by used a ratio of 1:2 [14], ie 2 kg of shrimp waste added with EM 1000 mL, aquades 3000 mL, and then mixed, fermented for 14 days under the anaerobic condition. After fermented, the liquid fertilizer the shrimp farming waste was filtered and sterilized. Nitrogen and phosphorus content
were analysis, then the results of the nitrogen and phosphorus tests were used in calculation in the addition of urea for make N P ratio for treatment.

2.3. Sterilization materials and equipment
Sterilized the device using liquid soap then rinse with distilled water, sterilized the aeration device soaked in 20 ppm chlorine for 24 hours, and neutralized with Na-thiosulfate [15]. Shrimp liquid waste fertilizer is put into a measuring flask then covered with cotton and wrapped in plastic wrap. Shrimp aquaculture liquid fertilizer is sterilized using a presto pan instead of an autoclave for 45 minutes. A pressure cooker has the principle of working together with the working principle of an autoclave, the optimal time to kill bacteria using presto is 45 minutes [16]. Sterilization of sea culture water media with 60 ppm chlorine for 24 hours, then neutralized with Na-thiosulfate at a dose of 20 ppm [17]. Media and equipment sterilization is done to avoid contaminants that can damage the cultivation activities [15].

2.4. Culture media
This research used media seawater 500 ml with salinity 31 ppt. Seawater then fertilized with the specified volumes Walne fertilizer (1 ml/L) and liquid fertilizer the shrimp farming waste with different N:P ratio (7 ml/L; 3.9 ml/L; 2.6 ml/L; 2.03 ml/L; 1.6 ml/L) and The environment in the culture media must be in optimal conditions.

2.5. Spreading inoculans
*N. oculata* obtained from Ugo Plankton Purworejo with a density of 10.7x10^6 cells/mL. The research used the initial density of *N. oculata* 100,000 cells/mL [18] Calculation of inoculum stocking volume uses the formula:

\[
V_1 = \frac{V_2 \times N_2}{N_1}
\]

Note :  
- \(V_1\) = Volume of the initial stocking (mL)  
- \(N_1\) = Density of stock *N. oculata* (cells/mL)  
- \(V_2\) = Volume of the culture medium (L)  
- \(N_2\) = Density of *N. oculata* culture (cells/mL)

2.6. Cultivation of Nannochloropsis oculata
*N. oculata* Culture in this research was conducted for 7 days. The purpose of culture was to determine the growth of *N. oculata* until a decline phase. The stability of water quality should be maintained in culture, the salinity, temperature, light intensity, supply oxygenated and pH was observed and tested every days.

2.7. Population and Growth Rate Calculation of Nannochloropsis oculata
Density of *N. oculata* cell calculated used Haemocytometer were observed under microscope binocular with a magnification of 100x, then calculated with formula [19]:

\[
\text{Density cells/ml} = \frac{n_a + n_b + n_c + n_d + n_e}{5 \times 4} \times 10^6
\]

- \(n_a, n_b, n_c, n_d, n_e\) = Total of *N. oculata* cells in the box a, b, c, d, e  
- \(\text{constans 5} = \text{Total of boxes counted}\)  
- \(4 \times 10^6\) = Area small box of a, b, c, d, e

Growth rate calculation with formula [20]:
The rate of growth (cell/ml/day) : $\frac{ln N_t - ln N_0}{\Delta t}$

Nt = Density N. oculata at the peak of the exponential phase (cell/mL)
N0 = Density N. oculata at the start of culture (cell/mL)
\Delta t = Difference in cell count time (days)

2.8. Data analysis
Data of population and growth rate N. oculata were analysed using One Way Analysis of Variance (ANOVA) and if was significant, tested further by Duncan’s Multiple Comparison Test (DMRT).

3. Result and discussion
3.1. Result
Shrimp fertilizer enriched with urea which was used as the cultivation medium of N. oculata produced a significant difference (<0.05) on the population and growth rate of N. oculata. The test results with Duncan showed that the shrimp fertilizer waste with different N: P ratios had a significantly different effect on each treatment. The first day P2 with a ratio of 10.5:1 produces the highest population, while P5 with an N: P ratio of 25.5:1 produces the lowest average population value. The second and third days P3 ratio 15.5:1 has the highest average population, and P5 ratio of 25.5:1 produces the lowest population which is not significantly different from P4 ratio of 20.5:1. On the fourth day of the highest average density at the N: P ratio was 15.5:1 and not significantly different from the P2 N:P ratio was 10.5:1, while the lowest average population density was at the N: P ratio of 25.5:1.

The highest peak of population on the fifth day is the N:P ratio of 15.5:1 with a density of 965x10^4 cells/ml and not significantly different from the ratio of 17:1 with an average density of 966x10^4 cells/ml. Whereas the N:P ratio of 25.5:1 resulted in the lowest population peak with an average density of 380x10^4 cells/ml and significantly different from other treatments. The highest average density of N. oculata on the sixth and seventh days in the N:P ratio of 15.5:1 which was not significantly different from the N: P ratio of 17:1 and significantly different from other treatments. The lowest population of the sixth and seventh days at the N: P ratio is 25.5:1. Maintenance for seven days the N: P ratio 15.5:1 was not significantly different from the N:P ratio 17:1, the N:P ratio 5.9:1 was not significantly different from the N:P ratio 10.5:1 on the fifth day until the seventh. The N:P ratio of 10.5:1 was significantly different on the first and fourth days with an N:P ratio of 15.5:1, while the N: P ratio of 20.5:1 was significantly different in the average cell density with an N: P ratio of 25.5:1 on the fourth and fifth days.

Table 2. The average growth population Nannochloropsis oculata with ratio N/P different

| Treatment | The ratio of N:P | P0 17:1 | P1 5.9:1 | P2 10.5:1 | P3 15.5:1 | P4 20.5:1 | P5 25.5:1 |
|-----------|------------------|---------|---------|----------|----------|----------|----------|
| 0 day     |                  | 10      | 10      | 10       | 10       | 10       | 10       |
| 1st day   |                  | 117±9a  | 75±3.8b | 105±8a   | 102±21a  | 58±10bc  | 50±5c    |
| 2nd day   |                  | 278±31a | 189±13b | 190±20b  | 316±22a  | 156±44bc | 128±10c  |
| 3rd day   |                  | 571±75abc| 434±98c | 465±7bc  | 618±81a  | 248±94d  | 214±27d  |
| 4th day   |                  | 813±87a | 594±160ab| 682±26ab | 830±86a  | 454±30d  | 294±42d  |
| 5th day   |                  | 966±65a | 700±162b| 795±23b  | 965±68a  | 525±15e  | 380±57d  |
| 6th day   |                  | 839±35a | 590±162b| 585±38b  | 792±79a  | 383±37c  | 281±83c  |
| 7th day   |                  | 734±16a | 488±165b| 436±57b  | 570±92abc| 225±65c  | 208±85c  |

The highest average growth rate on the first day was the N: P ratio of 10.5:1 of 95x10^4 cells/mL and was not significantly different from the N: P ratio of 15.5:1 and 17:1. The average growth rate the highest at second day was the N:P ratio 15.5:1 which was not significantly different from the N:P
ratio 17:1, while P5 with N:P ratio of 25.5:1 produced the lowest average growth rate and not significantly different from the N:P ratio 5:9:1. The highest growth rate on the third day is the N: P ratio of 15:5:1 with an average growth rate of 301x10^4 and not significantly different from the N:P ratio of 17:1, 5:9:1, 10:5:1. The lowest growth rate at the N:P ratio is 25.5:1 with an average growth rate of 85x10^3 and not significantly different from the N:P ratio of 20:5:1.

The highest average growth rate of *N. oculata* on the fourth day was the N:P ratio of 10:5:1 and was not significantly different with a ratio of 5:9:1, 15:5:1, 17:1 and 20:5:1. The highest average growth rate in fifth day of *N. oculata* at the N:P ratio 15.5:1 was not significantly different from the N:P ratio 17:1, 5:9:1, 10:5:1, but significantly different from the ratio N:P 20:5:1 and 25.5:1. The results of transformation data of the growth rate of *N. oculata* are shown in Table 3.

**Table 3.** The Average Growth Rate of *Nannochloropsis oculata* with Different of N:P Ratio

| Treatment | The average rate of *Nannochloropsis oculata* x 10^3 (cells/ml/day) ±SD |
|-----------|-------------------------------------------------|
|           | (17:1)                                         |
| 1st day   | 107±9^a                                        |
| 2nd day   | 160±40^ab                                      |
| 3rd day   | 293±63^a                                       |
| 4th day   | 241±12^a                                       |
| 5th day   | 153±25^a                                       |
|           | (5:9:1)                                        |
| 1st day   | 65±3,8^b                                       |
| 2nd day   | 113±14^bc                                      |
| 3rd day   | 245±85^a                                       |
| 4th day   | 160±70^b                                       |
| 5th day   | 106±29^bc                                      |
|           | (10:5:1)                                       |
| 1st day   | 95±8^a                                         |
| 2nd day   | 94±27^bc                                       |
| 3rd day   | 275±21^a                                       |
| 4th day   | 216±22^ab                                      |
| 5th day   | 112±6^bc                                       |
|           | (15.5:1)                                       |
| 1st day   | 92±21,7^a                                      |
| 2nd day   | 214±42^a                                       |
| 3rd day   | 301±70^a                                       |
| 4th day   | 211±12^ab                                      |
| 5th day   | 135±31^ab                                      |
|           | (20:5:1)                                       |
| 1st day   | 48±10,1^bc                                      |
| 2nd day   | 98±53^bc                                       |
| 3rd day   | 91±50^b                                        |
| 4th day   | 205±65^ab                                      |
| 5th day   | 71±20^c                                        |
|           | (25:5:1)                                       |
| 1st day   | 40±5^c                                         |
| 2nd day   | 78±12^c                                        |
| 3rd day   | 85±17^b                                        |
| 4th day   | 80±15^c                                        |
| 5th day   | 86±15,2^c                                      |

3.2. Discussion

The different N: P ratios of shrimp fertilizer waste affect the population and growth rate of *N. oculata*. The N: P ratio affects the availability of nutrients in the media, the elements N and P are limiting factors for microalgae [21]. Nitrogen is an essential element for the growth of microalgae because it affects the composition of protein structures such as enzymes, genetic material, peptides, and chlorophyll in microalgae cells [22]. Whereas phosphorus plays a role in the formation of ATP and the formation of nucleic acids namely DNA and RNA [23]. The limitation of N and P elements can cause the protein content in microalgae cells to decrease through degradation of various cell components [6].

The growth of *N. oculata* with culture fertilizer media of shrimp culture waste with different N:P ratios experienced four phases as found in plankton culture, namely adaptation, exponential, rate reduction, stationary and death phases [24]. Four phases, namely the adaptation phase, occurs after the spread of *N. oculata* inoculants into the culture media. The first day to the fifth day is an exponential phase, until the fifth day which is the peak of population growth. The fourth day to the fifth day is a slowdown phase. The death phase occurs on the sixth and seventh day.

Shrimp culture waste fertilizer containing 215.42 mg/L and 36.19 mg/L phosphorus with an N:P ratio of 5.9:1. Increasing the N:P ratio of liquid fertilizer to aquaculture is done by adding urea as a nitrogen source. Liquid fertilizer of shrimp culture waste with an N: P ratio of 15.5:1 produces the highest population on the fifth day. The same thing was also reported by Rasdi and Qin [9], that the optimal requirement for N:P ratio *N. oculata* ranges from 10-20:1. The peak population of *N. oculata* which is cultivated using liquid fertilizer of shrimp culture waste with an N: P ratio of 15.5:1 produces an average density of 965x10^4 cells/ml not significantly different from walne fertilizer with an N: P ratio of 17:1. This is in accordance with the opinion of Hidayati [8] that the liquid fertilizer of shrimp aquaculture used for the cultivation of *Chlorella vulgaris* at an N:P ratio of 10:1 produces an average density of 1030x10^4 cells/ml.

Population density of *N. oculata* which is cultivated using liquid fertilizer of shrimp culture waste with an N:P ratio of 15.5:1 with an average density of 965x10^4 cells/ml is higher than the population of *N. oculata* which is cultivated using liquid fertilizer *Gracilaria* sp. with an N:P ratio of 1.4:1 with an average density of 695x10^4 cells/ml [25]. The growth of *N. oculata* is influenced by several factors namely water quality and the environment of cultivation media, the availability of macronutrients and micronutrients in the media, the quality of *N. oculata* seedlings, the concentration of N and P values.
Water quality of \textit{N. oculata} culture media includes temperature 25.7-29°C, pH 7-8.5, salinity 30-35 ppt, and light intensity 5.010-5.622 lux. Water quality in the culture media using liquid fertilizer shrimp shrimp waste according to the needs of \textit{N. oculata} namely temperatures 25-32°C [27], pH 7-9.4 [28], 25-45 ppt [29], light intensity 2000-8000 lux [30]. The factor influencing the growth of \textit{N. oculata} in this study is the ratio of N:P fertilizer for shrimp culture waste.

The use of liquid shrimp fertilizer waste using a different ratio of N:P ratios has an effect on the growth rate of \textit{N. oculata}. The third day of growth \textit{N. oculata} achieved the highest growth at an N:P ratio of 15.5:1 with an average density of 301x10^4 cells/ml/day. The growth rate of \textit{N. oculata} on the third day entered the exponential phase. The exponential phase is characterized when an increase in cell population density is one-fold or more than the initial density [31]. The faster of the growth rate, the better of the carrying capacity of the fertilizer media to the growth of \textit{N. oculata} [32]. Decrease in growth rate occurs on the fourth day to the fifth day, this is due to competition between cells of \textit{N. oculata} to fight nutrients limited to the media [33].

\textit{N. oculata} in this study was cultivated using shrimp culture waste with the same nitrogen concentration in all treatments and different phosphorus concentrations, resulting in different population growth and growth rates. The use of an N: P ratio that is higher and lower than the N: P ratio of 15.5:1 on enriched with urea shrimp culture waste results in lower population and growth rates. \textit{N. oculata} cultivation using shrimp waste fertilizer was ratio of N:P 25.5:1 resulted in the lowest growth rate of 85.8x10^4 cells/day. The growth of \textit{N. oculata} can be inhibited due to the element phosphorus [34]. The availability of phosphorus nutrients is very necessary for the ongoing process of cell metabolism and energy transfer for the survival of microalgae [35]. But the availability of phosphorus is too high can cause excessive phytoplankton growth [36].

4. Conclusion
The use of shrimp fertilizer waste with a ratio of N:P 15.5:1 produces in the highest growth population namely an average of 965x10^4 cells/mL and an average growth rate of 135x10^6 cells/ml/day.

5. References
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