

**First report of *Spirulina* sp. performance in wastewater of *Cromileptes altivelis* aquaculture in Indonesia**

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**Abstract**. Indonesia is one of the countries with the most significant grouper production in the world. One of the groupers that are widely cultured in Indonesia is the humpback grouper (*Cromileptes altivelis*). Unfortunately, humpback grouper aquaculture activities cause waste. The waste can produce hydrogen sulfide, orthophosphate, and ammonia, which is toxic to aquatic animals. One way to treat the waste is by using *Spirulina* sp. *Spirulina* sp. is utilizing organic material from humpback grouper waste to support its growth. The purpose of this study was to identify its performance in reducing water quality parameters into tolerance level. It was also to examine the growth of *Spirulina* sp. on laboratory-scale culture in the humpback grouper nursery media with a varying degree of waste. The study design used was a Completely Randomized Design (CRD) with four treatments and three replications, A (100%), B (75%), C (50%), and D (25%) humpback grouper nursery waste as culture media. The parameters calculated include population density, nitrate, orthophosphate, pH, temperature, salinity, and light intensity. The results showed that *Spirulina* sp. could reduce nitrate and phosphorus in water up to 89.78% and 84.34% of the waste. *Spirulina* sp. could maintain all water quality to be following the standards for fish farming again. The utilization of humpback grouper nursery waste at a concentration of 25% has a significant effect on the population growth of *Spirulina* sp. on laboratory scale culture.

**Key Words**: *Cromileptes altivelis, Spirulina*, wastewater, aquaculture, Indonesia.

**Introduction**. Indonesia is one of the countries with the most significant grouper production in the world. Together with China and Taiwan, Indonesia produces a total of 92% of grouper production (Rimmer & Glamuzina 2019). Grouper production in Indonesia can reach more than 10,200 tons in 2012 and continues to increase each year (Mutalib & Khartiono 2018). Grouper fish production in Indonesia is supported by the diversity of grouper species spread throughout the region. Earlier reports mentioned that grouper types found in Indonesia such as napoleon wrasse (*Cheilinus undulatus*), leopard coral trout (*Plectropomus leopardus*), estuary cod (*Epinephelus coioides*), areolate grouper (*Plectropomus areolatus*), brown marbled grouper (*Epinephelus fuscoguttatus*), and humpback grouper (*Cromileptes altivelis*) (Khasanah et al 2019). Other reports that several hybrid groupers were successfully developed in Indonesia (Murwantoko et al 2019).cThe high production of grouper is due to its high prices demand. Grouper has characteristics of good taste, fast growth, high adaptability, easy and understandable to be cultured (Tian et al 2017; Chen et al 2018; Megarajan et al 2015; Cai-Juan et al 2016; Soong et al 2016). Therefore, grouper production is mostly done, especially in the field of aquaculture.

One of the groupers that are widely cultivated in Indonesia is the humpback grouper (*Cromileptes altivelis*). Humpback groupers are Indonesia's mainstay commodity (Ansari et al 2016). Humpback grouper is found in almost all marine areas such as Banten Bay, Riau, Ujung Kulon, Seribu Islands, Madura, Nusa Tenggara, and Kalimantan (Tridjoko et al 2017). Humpback grouper aquaculture in Indonesia has been developed for a long time and achieved rapid progress (Dody & La Rae 2016). Unfortunately, humpback grouper aquaculture also has adverse effects due to its waste.

Waste generated from grouper aquaculture can be either organic or inorganic matter. Organic matter is caused by metabolic wasters, feces, uneaten feeds, and residues of medications used (Herath & Satoh 2015). Dissolved water production consisting of N and P will increase due to the presence of leftover food and feces (Dauda et al 2018). Reforming these organic materials will produce hydrogen sulfide, orthophosphate, and ammonia, which are toxic to fish (Dia et al 2018). In addition to ammonia, organic matters left in the waters can be reformed into orthophosphate (Li et al 2019), while inorganic materials are caused by the use of antibiotics and drugs (Edwards 2015). This inorganic material can cause eutrophication and pharmaceuticals’ pollution (He et al 2016). This waste is dangerous if not treated first because it can spread the disease (Boerlage et al 2017). Waste can also cause pollution and threaten sustainability, even mortality (White et al 2017).

One method that can be used to overcome the waste is by using *Spirulina* sp. *Spirulina* sp. is plankton characterized by its spiral-shaped, filamentous, and multicellular photosynthetic blue-green algae (Mostafa & El-Gendy 2017). Recently, *Spirulina* sp. is widely used in waste management (Zheng et al 2017). *Spirulina* sp. can remove heavy metals in the waters (Balaji et al 2015). Even *Spirulina* sp. has been used to handle the aquaculture waste (Wuanget al 2016). The ability of *Spirulina* sp. to cope with waste is due to its ability to utilize the nitrogen in the form of nitrate and phosphate in the form of orthophosphate to support its growth (Shanthi et al 2018). Besides being able to get rid of waste content, *Spirulina* sp. also has many advantages, such as fast-growing and quickly produced (Singh % Parwani 2018). *Spirulina* sp. also doesn't need fertile or full area (Rempel et al 2019).

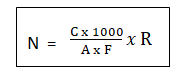
In previous studies, *Spirulina* sp. can reduce the content of ammonia, nitrate, nitrite, and phosphate up to 94.8% (Nogueira et al 2018). In Indonesia, *Spirulina* sp. has been used to treat waste produced from Pangasius farming (Wijayanti et al 2019). However, the *Spirulina* sp. utility for grouper waste treatment has not been reported. Therefore, this study aims to determine the results of *Spirulina* sp. performance to treat the humpback grouper waste, one of the fish with high economic value, to conform to existing standards in Indonesia.

**Material and Method**

***Material of the study***. This study was conducted at the Aquaculture Laboratory, Department of Fisheries and Marine Science, Faculty of Agriculture, University of Lampung in 2019. *Spirulina* was obtained from Research Center and Development of Marine Aquaculture, Pesawaran, Lampung Province, Indonesia. Preparation of inoculant *Spirulina* began by preparing a 3-liter glass jar filled with 2 liter of seawater and 2 ml of Conway fertilizer (1mL/L dose). 200-400 mL inoculant seedlings (10-20% of the water volume) were added and aerated, covered in jars and cultured for 5 days to be ready to be used as a laboratory-scale inoculant stock. The laboratory scale temperature was set at 20°C and 36 watt TL lamp with a 24-hour irradiation time per day and aeration.

***Experimental design***. This study used a Completely Randomized Design (CRD) consisting of four treatments and three replications. The treatment used different concentrations of sewage and sterile seawater, as follows: treatment A (100% sterile humpback grouper nursery waste), treatment B (75% sterile humpback nursery waste + 25% sterile seawater), treatment C (50% sterile humpback nursery waste + 50% sterile seawater), treatment D (25% sterile humpback nursery waste + 75% sterile seawater). Measured parameters in this study were *Spirulina* sp. growth and water quality (nitrate, phosphate, temeprature, pH, salinity and light intensity).

***Spirulina* sp*.growth measurement***. The initial density calculation of *Spirulina* sp*.*  was carried out to determine the density of the inoculum to be used in a culture bottle. Initial individual density was calculated using Sedgewick rafter with three replications. Inoculant of *Spirulina* has put into each culture bottle as much as 100 mL in 300 mL media. The calculation was conducted using the formula from the previous study (Wicaksono et al 2019) as follows:



Explanation:

N: Density of *Spirulina* sp. (ind/mL)

C: Number of individuals counted

A: Constants (π)

R: Dilution

F: Number of fields of view

***Measurement of Water Quality.*** Water quality measured in this study were nitrate, phosphate, temeprature, pH, salinity and light intensity. Nitrate measurement was carried out using the SNI 06-2480-1991 method using a pyrosulfate spectrophotometer at a wavelength of 410 nm (Nandiyanto & Haristiani 2017). Nitrate analysis was done every three days during the study. Phosphate measurement was carried out using the SNI 06-6989.31-2005 method with a spectrophotometer and ascorbic acid utilization at a wavelength of 880 nm (Dewi et al 2019). Phosphate analysis was done every three days during the study. Observations on the physical parameters of water were temperature and light intensity, while the chemical water parameters were pH, salinity, nitrate, and phosphate. Temperature, pH, and salinity were measured every 24 hours using a thermometer. Observations made on the parameters of water biology are observations on population densities of *Spirulina* sp. by calculating population density once every 24 hours starting from the first day (T0) until the end of the study (T9).

***Data analysis****.* This study uses a test for normality and homogeneity of data as well as analysis of variance (ANOVA) at a 95% confidence level. After the data is known to have a significant effect, then proceed with the least significant difference test (LSD) with a confidence level of 95% to find out significant differences between treatments.

**Results and Discussion**. *Spirulina* sp. growth during treatment of grouper wastewater

showed in Fugre 1. In day 1, the culture began with the highest number in treatment B with a density of 0.1573.106, followed by C (0.1572.106), A (0.156.106), and D (0.1467.106). On the second day, all treatments experienced a decrease in the amount of density. In treatment A with a density of 0.054.106 ind/mL, treatment B with a density of 0.047.106 ind/mL, treatment C with a density of 0.045,106 ind/mL, and treatment D with a density of 0.042,106 ind/mL. This was because *Spirulina* sp. experienced a lag phase, where plankton adjusted to their new environment (Da Silva Braga et al 2019). In this phase, plankton synthesizes the enzymes needed to metabolize the compounds that existed in the media (Soccol et al 2016). Slowing algal growth occurred in this phase because the energy possessed by *Spirulina* sp., was focused on adjusting itself to new culture media and for maintenance so that it is not widely used for growth (Fogg 1975).

Figure 1. *Spirulina* sp. growth during treatment of grouper wastewater

After the adaptation phase/lag phase ended, the growth of *Spirulina* sp., entered the exponential phase. In this study, the exponential phase occurred on days 3 to 5 (for treatment A) and 6 (treatments B, C, D). In this phase, *Spirulina* sp. would experience rapid growth (Puspanadan et al 2018). This was because of *Spirulina* sp. cells had adapted to the environment (Moreira et al 2016). *Spirulina* sp. would undergo division, the division of these cells caused the growth of *Spirulina* sp., sprinted in the exponential phase (Umainana et al 2019). During the exponential phase, an event called doubling time occurred, which was dead cells called nikrida would break up immediately. Trichome would be fragmented into a cell colony called hormogonium (Vaz et al 2004). After that, it separated from its parent filament to become a new trichome cell (Ciferri 1983). The population of *Spirulina* sp., in treatment D with a density of 2.66.106 ind/mL, treatment C with a density of 1.85.106 ind/mL, treatment B with a density of 1.81.106 ind/mL and treatment A with a density of 0.74.106 ind/mL on the day 7th. The highest density (peak) of *Spirulina* sp. culture occurred on the 6th day of treatment A with a density of 1.68.106 ind/mL while on the day 7 for treatment B, C, D with consecutive densities of 1.81.106 ind/mL, 1.85.106 ind/mL and 2.66.106 ind/mL. After the end of the peak phase, the culture population experienced a death phase. Interestingly, in treatment A with a density of 0.54.106 ind/mL occurred in day 7, whereas in treatment B with a density of 1.09.106 ind/mL, treatment C with density 1.11.106 ind/mL, treatment D with density 1.68.106 ind/mL, occurred at day 8. This was due to treatment A having the highest grouper wastewater cultivation content. High waste content indicated the presence of nutrients for *Spirulina* sp. and an increase in the number of algal populations in the media accelerated the reduction of nutrients in the media (Kalsum et al 2019). Treatment D produced the most biomass. This was because treatment D had the lowest waste content. The content of waste in the media could be a stress compound for *Spirulina* sp., so the amount of biomass was decreasing.

***Nitrate and phosphate reduction***. Nitrate levels data could be seen in Figure 2. On the 9th day, treatment A had the highest nitrate levels of 4.13 mg/L, compared to treatments B, C and D, each of which was 3.31 mg/L, 1.61 mg/L and 0.38 mg/L. A very significant decrease in nitrate levels occurred in treatment D with a decrease of 3.34 mg/L at the beginning of culture (day 0) and end of culture (day 9) with a percentage reduction of 89.78%. The lowest decrease occurred in treatment A with a decrease of 3.06 mg/L at the beginning of culture (day 0) and end of culture (day 9). The results of statistical tests on the reduction of nitrate levels showed a significant effect on the growth of *Spirulina* sp. on laboratory scale culture in the humpback grouper media (p <0.05). The Least Significant Difference (LSD) test results showed that between all treatments were significantly different.

# Figure 2. Nitrate content of during treatment of grouper wastewater.

Phosphate levels data showed in Figure 3. The lowest level of phosphate was in treatment D with value of 1.14 mg/L compared with the treatments A, B and C, each of which was 5.88 mg/L, 4.23 mg/L and 1.14 mg/L. A very significant decrease in phosphate levels occurred in treatment D, which was able to reduce phosphate levels by 6.14 mg/L with a reduction percentage of 84.34%. But on the contrary, the lowest decrease occurred in treatment A, which was able to reduce phosphate levels by 4.34 mg/L with a decrease percentage of 42.47%. Statistical test results indicated that the decrease in phosphate levels showed a significant effect on the distribution of humpback grouper nursery waste with different concentrations on the growth of *Spirulina* sp. population on laboratory scale culture (p<0.05). The Least Significant Difference (LSD) test results showed that treatment A was significantly different from all treatments, treatment B was not significantly different from treatment C and treatment D was significantly different from all treatments.

Figure 3. Phosphate content of during treatment of grouper wastewater.

After nine days, *Spirulina* sp. succeeded in reducing nitrate content in grouper aquaculture waste. Nitrate and phosphate levels at the end of *Spirulina* sp. culture measurements appeared to be reduced compared to nitrate and phosphate levels at the beginning of the analysis. This indicated that the absorption of nitrate and orthophosphate by *Spirulina* sp., met nutritional needs. This was consistent with previous research that *Spirulina* sp. could process nitrate and phosphate content in fishery waste (Askari Hesni et al 2019). The ability of *Spirulina* sp. in reducing nitrate by utilizing nitrate as the main component in water actived to support its growth (Manirafasha et al 2018). *Spirulina* sp. had nitrate enzymes such as nitrate reductase, which functioned to utilize nitrates in the media for growth and metabolism (Esen & Urek 2015).

Phosphorus was a nutrient needed by microalgae for the synthesis of nucleic acids and high energy compounds for growth (Mehar et al 2019). In this study, *Spirulina* sp. could reduce nitrate up to 89.78% in treatment D, but the lowest decrease occurred in treatment A with 42.56%. Previous studies had explained that the smaller the nitrate content in feeding waters, the higher the ability to remove nitrates (Lodi et al 2003). The lowest level of phosphate in treatment D was due to the high population density of *Spirulina* sp. in the medium, so the utilization of phosphate was high (Çelekli et al 2009). This study also indicated that the higher the concentration of humpback grouper waste was given, the higher the turbidity level. High turbidity levels caused low light intensity and would create a decrease in phosphorus removal (Markou et al 2012). The value of initial, final and reduction percetage of nitrate and phosphate obtained in the treatment showed in Table 1.

Table 1. Value of initial, final and reduction percetage of nitrate and phosphate obtained in the treatment.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameter | Treatment | | | | | | | | | | | |
| A | | | B | | | C | | | D | | |
| Initial | Final | Reduction | Initial | Final | Reduction | Initial | Final | Reduction | Initial | Final | Reduction |
| Nitrate (mg/L) | 7.19 | 4.13 | 42.56% | 6.17 | 3.31 | 46.35% | 4.83 | 1.61 | 66.67% | 3.72 | 0.38 | 89.78% |
| Phosphate (mg/L) | 10.22 | 5.88 | 42.47% | 9.15 | 4.23 | 53.77% | 9.02 | 3.57 | 60.42% | 7.28 | 1.14 | 84.34% |

Nitrate reduction from *Spirulina* sp. had met Indonesian standards. Nitrate content recommended for fish farming in Indonesia was 10 mg/L (Tatangindatu et al 2013). Nitrate content following the standards caused the fish to grow optimally. If nitrate was not controlled, it could influence total hemoglobin and blood glucose, creating slow growth, natural pain, low FCR to death (Waikhom et al 2018).

While for phosphate content, only treatment D met the culture standards in Indonesia with values below 0.2 mg/L Phosphate (Pagoray & Ghitarina 2016). This was because treatment D had the lowest waste content (25%). High phosphorus content would reduce protein intake to fish, leading to potentially harmful organisms that could cause environmental pollution and even death in fish (Lim et al 2018).

***Water quality measurement*.** Important factors influencing the growth of microalgae were water quality, such as the physical and chemical conditions of the water from the medium (Tinambunan et al 2017). Physical conditions included temperature and light intensity while chemical conditions included salinity and pH (Suthers et al 2019). The environmental factors measured in this study were temperature, degree of acidity (pH), salinity and light intensity. The values of other water quality parameters could be seen in Table 2.

Table 2. Water parameter quality of temperature, pH and light intensity.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameter | Treatment | | | | | | | | Standard |
| A | | B | | C | | D | |
| Initial | Final | Initial | Final | Initial | Final | Initial | Final |
| Temperature (oC) | 25 | 23 | 25 | 23 | 25 | 23 | 25 | 23 | 20-30 |
| pH | 7.53 | 8.87 | 7.37 | 8.9 | 7.4 | 8.93 | 7.73 | 8.87 | 7-11 |
| Salinity (ppt) | 33 | 39.67 | 33 | 40.67 | 33 | 41 | 33 | 41.33 | 30-60 |
| Light Intensity (lux) | 4143.33 | 3183.67 | 4017 | 3209.67 | 4162 | 3323.33 | 4179 | 3134.33 | 1500-4500 |

Source : a : Hariyati (2008)

b : Ciferri (1983)

c : Richmond (2008)

Temperature was an essential factor for the life of organisms because temperature significantly affected both metabolic activity and the development of microalgae (Li et al 2016). Based on observations at each treatment, the temperature in the water medium ranged from 23-25°C. The temperature in the study was still in the optimum temperature range for the growth of *Spirulina* sp., which was under the previous research statement (Hariyati 2008) that the optimum temperature for the growth of *Spirulina* sp. ranged from 20-30°C. The temperature range was due to the culture could perform better temperature control (Mahmoud et al 2016).

Another factor that was very influential on the growth of *Spirulina* sp. was pH (Moreira et al 2016). Controlling the pH of the medium was very important to maintain the growth balance of *Spirulina* sp., (Ismaiel et al 2016). The pH value in all treatments was still within the tolerance range of *Spirulina* sp. (Ciferri 1983). Interestingly, there was an increase in the pH value before and after the study. The rise in pH could be caused by the use of CO2 by *Spirulina* sp. as a carbon source for photosynthesis due to CO2 so that it induced pH change (Wang et al 2019).

Salinity played a vital role in the life of aquatic organisms that affected the growth rate because salinity stress would affect osmoregulation (carbohydrate metabolism) through consuming energy processes to overcome high salinity (Çelekli et al 2016). Based on observations in each treatment, salinity in the water medium ranged between 33-42 ppt and was still in the optimum salinity range for *Spirulina* sp. growth media (Richmond 2008). At each treatment, salinity in the water medium increased. Increased salinity could occur due to water evaporation which reduced the volume of water so that the concentration of dissolved salts in it increased (Yang et al 2017).

Light was an essential requirement for *Spirulina* sp. because it was a phototrophic organism that uses light as an energy source (De Fontoura Prates et al 2018). Based on observations in each treatment, the average light range was 3088-4298 lux, and the results were still in the field of optimal light intensity for the growth of *Spirulina* sp., which ranged between 1500-4500 lux (Richmond 2008).

**Conclusions**.This study had successfully confirmed the ability of *Spirulin*a sp. to remediate waste water from humpback grouper. *Spirulina* sp. is capable of reducing nitrate and phosphorus in water up to 89.78% and 84.34% of the waste, following the Indonesian standard. Not only notrate and phosphate, *Spirulina* sp. could maintain all water quality to be following the standards for fish farming again such as temperature, pH, salinity and light instensity. The utilization of humpback grouper waterwaste at a concentration of 25% had a significant effect on the population growth of *Spirulina* sp. on laboratory scale culture. This technique had many benefits because the produced *Spirulina* can be used as fish feed, beauty products, medicines, even biodiesel. This was possible due to the utilization of *Spirulina* as wastewater treatment which also improved the microalga production.

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