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., which 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 to 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: aquaculture, grouper, phytoplankton, waste.

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 reached 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 say that several hybrid groupers were successfully developed in Indonesia (Murwantoko et al 2018). The high production of grouper is due to its high prices demand. Grouper has the characteristics of good taste, fast growth, high adaptability and easiness 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 one of Indonesia's mainstay commodities (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 wastes, faeces, and uneaten feeds used (Herath & Satoh 2015). Dissolved water production consisting of nitrogen and phosphor will increase due to the presence of leftover food and faeces (Dauda et al 2018). Reforming these organic materials will produce hydrogen sulfide, orthophosphate, and ammonia, which are toxic to fish (Dai et al 2018). In addition to ammonia, organic matters left in the waters can be reformed into orthophosphate (Li et al 2019), while inorganic matter is produced by the use of antibiotics and drugs (Edwards 2015). This inorganic matter can cause eutrophication and pharmaceuticals' pollution (He et al 2016). This waste is dangerous if not treated first because it can spread diseases (Boerlage et al 2017). Aside from disease, both organic and inorganic wastes could threaten the environmental sustainability and causes pollution that often ends in 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). *Spirulina* sp. has been used to handle aquaculture waste (Wuang et 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 it is fast-growing and quickly produced (Singh & Parwani 2018). In terms of production, *Spirulina* sp. requires little space so it can be cultured in most areas (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 from 01 January to 28 February 2019. *Spirulina* sp. was obtained from the 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 liters of seawater and 2 ml of Conway fertilizer (1mL/L dose). 200-400 mL of 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 parameters: 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 the 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:

$$N = \frac{C \times 1000}{A \times F} x R$$

where:
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 parameters 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 multiparameter device (Hanna Instruments® HI 98195). 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, we proceeded 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 is displayed in Figure 1. In day 1, the culture began with the highest number in treatment B with a density of 0.1573 x 10^6, followed by C (0.1572 x 10^6), A (0.156 x 10^6), and D (0.1467 x 10^6). On the second day, all treatments experienced a decrease in density. In treatment A with a density of 0.054 x 10^6 ind/mL, treatment B with a density of 0.047 x 10^6 ind/mL, treatment C with a density of 0.045 x 10^6 ind/mL, and treatment D with a density of 0.042 x 10^6 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 prioritized to maintain itself and the whole enzymatic process. Less energy was used for growth, so it is quite lacking in this phase (Fogg 1975).

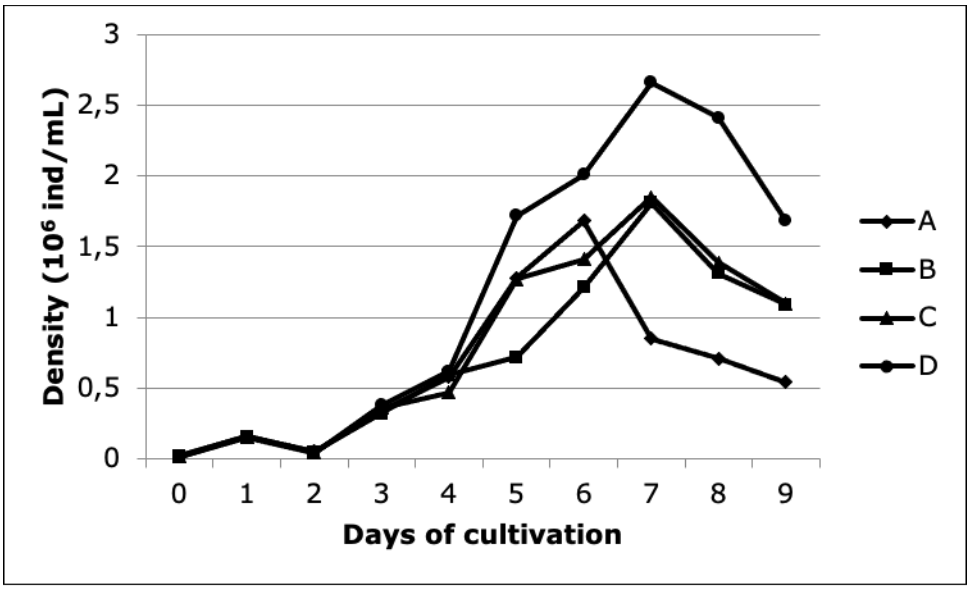

Figure 1. *Spirulina* sp. growth during treatment of grouper wastewater.

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. and sprinted the exponential phase (Umainana et al 2019). During the exponential phase, an event called doubling time occurred, which consists of dead cells called nikrida which would break up immediately. Trichomes 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 x 10^6 ind/mL, treatment C with a density of 1.85 x 10^6 ind/mL, treatment B with a density of 1.81 x 10^6 ind/mL and treatment A with a density of 0.74 x 10^6 ind/mL on the day 7th. The highest density (peak) of *Spirulina* sp. culture occurred on the 6^{th} day of treatment A with a density of 1.68 x 10^6 ind/mL and on the 7^{th} day for treatment B, C, D with consecutive densities of 1.81 x 10^6 ind/mL, 1.85 x 10^6 ind/mL, and 2.66 x 10^6 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 x 10^6 ind/mL occurred on day 7, whereas in treatment B with a density of 1.09.10^6 ind/mL, treatment C with density 1.11 x 10^6 ind/mL, treatment D with density 1.68 x 10^6 ind/mL, occurred on 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. The statistical difference bar is showed in Figure 2.

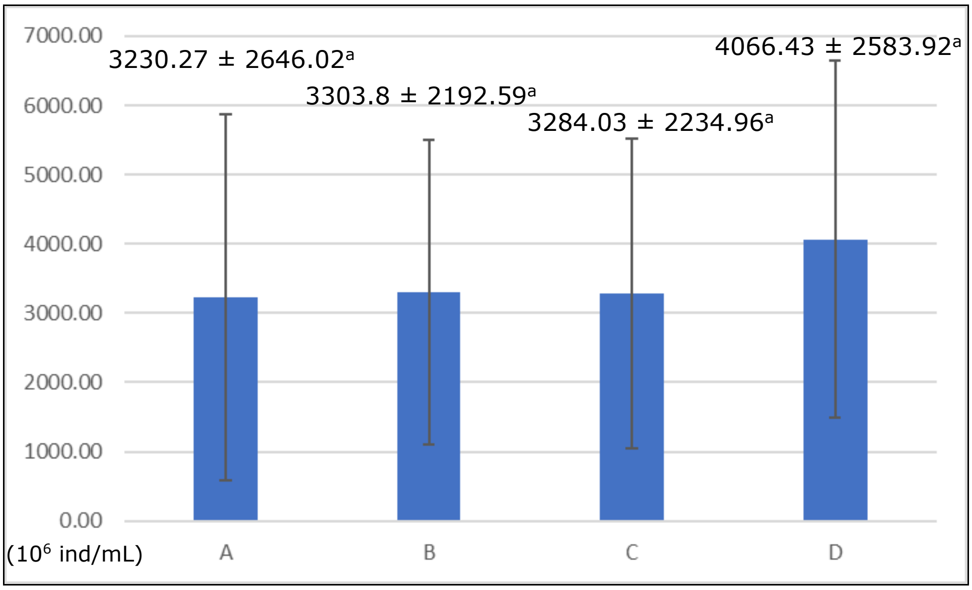

Figure 2. Statistical difference of *Spirulina* sp. growth during treatment.

Nitrate and phosphate reduction. Nitrate levels data can be seen in Figure 3. 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 the culture (day 0) and at the end of the 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 between all treatments are showed in Figure 4.

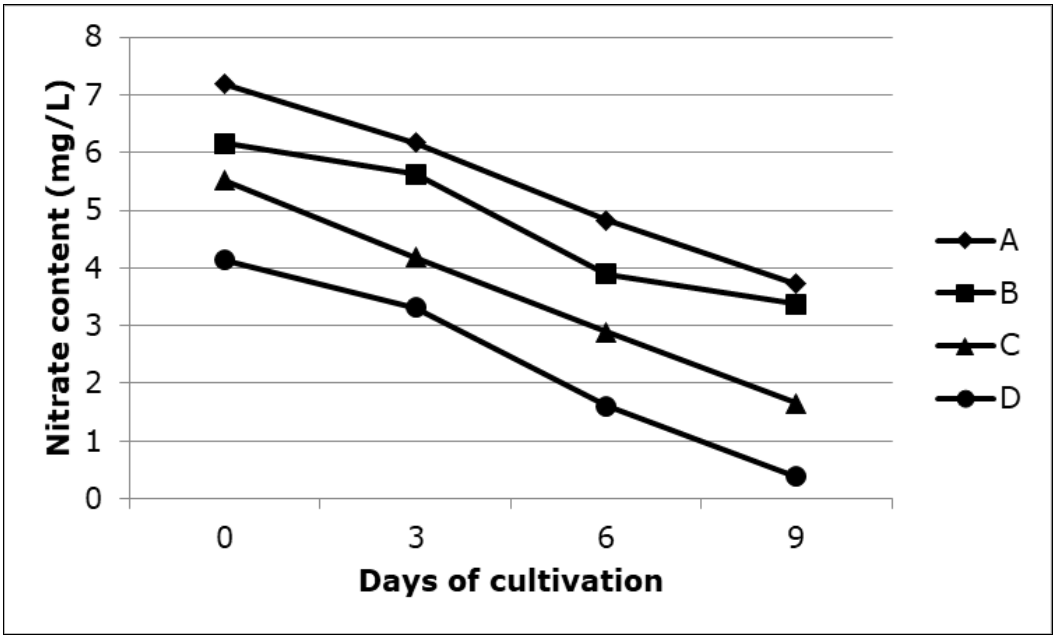

Figure 3. Nitrate content during treatment of grouper wastewater.

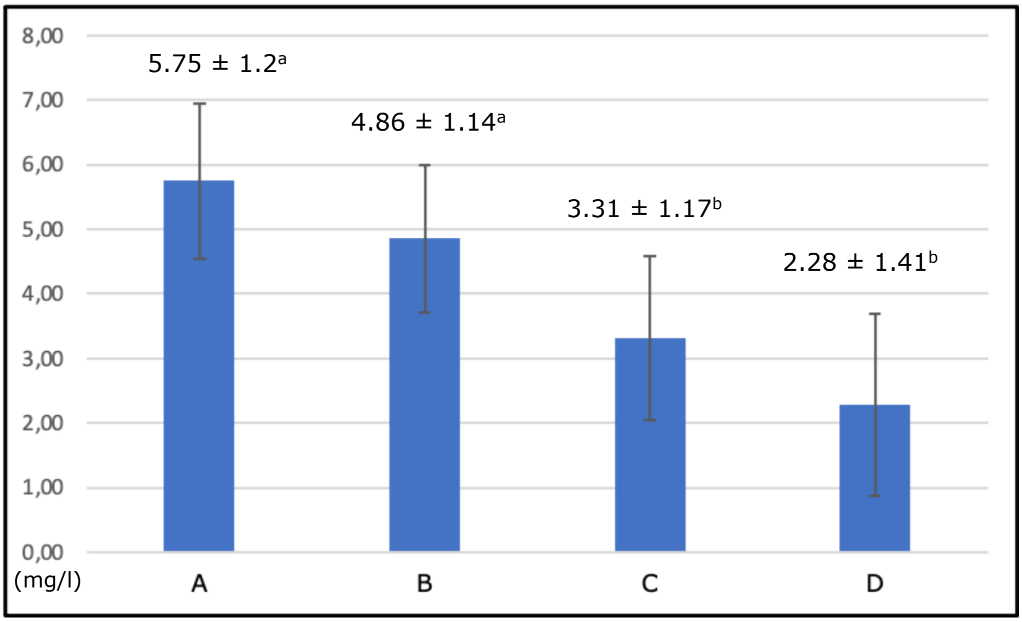

Figure 4. Statistical difference of nitrate content during treatment.

Phosphate levels data is displayed in Figure 5. The lowest level of phosphate was in treatment D with a 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 are showed in Figure 6.

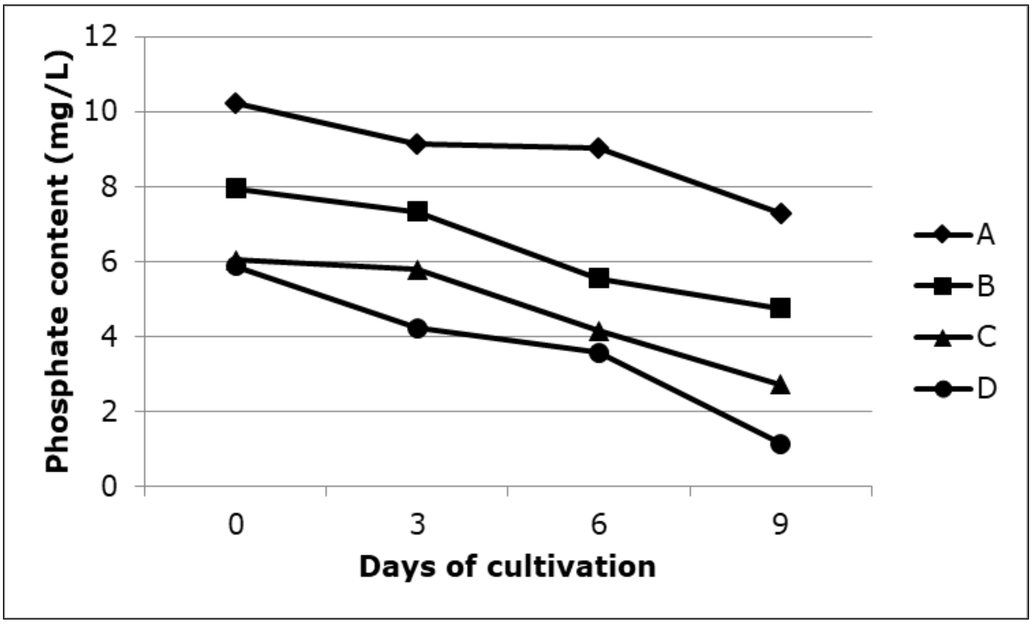

Figure 5. Phosphate content during treatment of grouper wastewater.

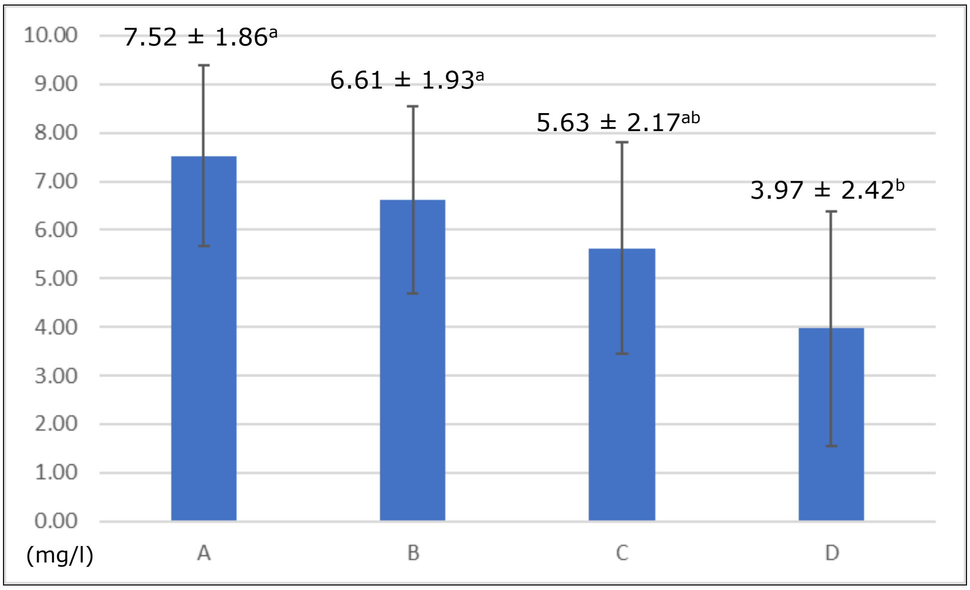

Figure 6. Statistical different of phosphate content during treatment.

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 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 is 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 are shown in Table 1. The statistical difference of nitrate and phosphate are presented in Figure 7 and Figure 8.

Table 1

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

Treatment		*Nitrate*	*Phosphate*
A	Initial (mg/L)	7.19[a]	10.22[a]
	Final (mg/L)	4.13 [a]	5.88 [a]
	Reduction (%)	42.56%	42.47%
B	Initial (mg/L)	6.17[b]	9.15 [b]
	Final(mg/L)	3.31 [b]	4.23 [b]

	Reduction (%)	46.35%	53.77%
C	Initial (mg/L)	4.83[c]	9.02[b]
	Final (mg/L)	1.61[c]	3.57[b]
	Reduction (%)	66.67%	60.42%
D	Initial (mg/L)	3.72[d]	7.28[c]
	Final (mg/L)	0.38 [d]	1.14[c]
	Reduction(%)	89.78%	84.34%

Note: Mean values in the same row with different superscript letters show significant differences between the groups ($p<0.05$).

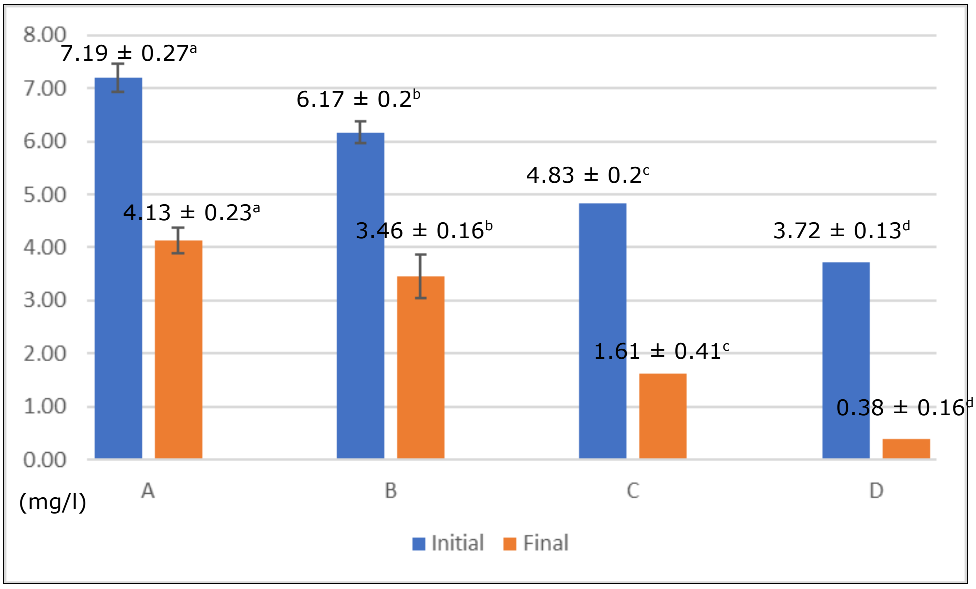

Figure 7. Statistical difference of initial and final value of nitrate obtained in the treatment (mean values in the same row with different superscript letters show significant differences between the groups ($p<0.05$)).

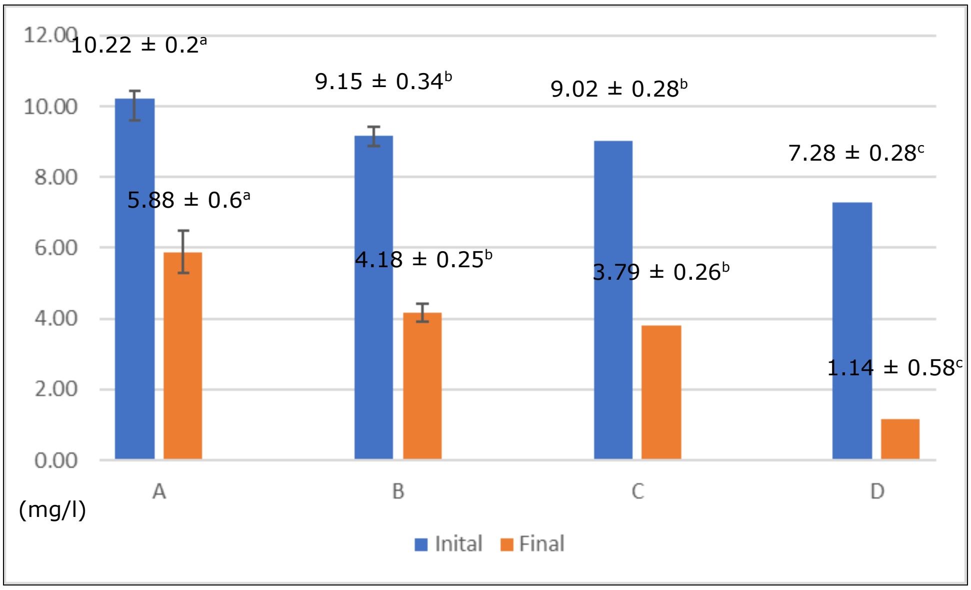

Figure 8. Statistical difference of initial and final value of phosphate obtained in the treatment (mean values in the same row with different superscript letters show significant differences between the groups ($p<0.05$)).

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 were not controlled, it could influence total hemoglobin and blood glucose, creating slow growth, natural pain, low feed conversion rate and 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 regarding the water quality are influencing the growth of microalgae, 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 can be seen in Table 2. The graphic bars of temperature, pH, salinity and light intensity were showed in Figures 9, 10, 11, and 12 respectively.

Table 2

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

Treatment		Temperature (°C)	pH	Salinity (ppt)	Light Intensity (lux)
A (Hariyati 2008)	Initial	25[a]	7.53[a]	33[a]	4143.33[a]
	Final	23[a]	8.87[a]	38.67 [a]	3183.67[a]
B (Ciferri 1983)	Initial	25[a]	7.37[a]	33.33[a]	4017 [a]
	Final	23[a]	8.9[a]	38[a]	3209.67 [a]
C (Richmond 2008)	Initial	25[a]	7.4[a]	34[a]	4162[a]
	Final	23[a]	8.93[a]	38.33[a]	3323.33[a]
D	Initial	25[a]	7.33[a]	34[a]	4179[a]
	Final	23[a]	8.87[a]	38.67[a]	3134.33[a]
Standard		20-30[A]	7-11[A]	30-60[B]	1500-4500[C]

Note: Mean values in the same row with different superscript letters show significant differences between the groups ($p<0.05$).

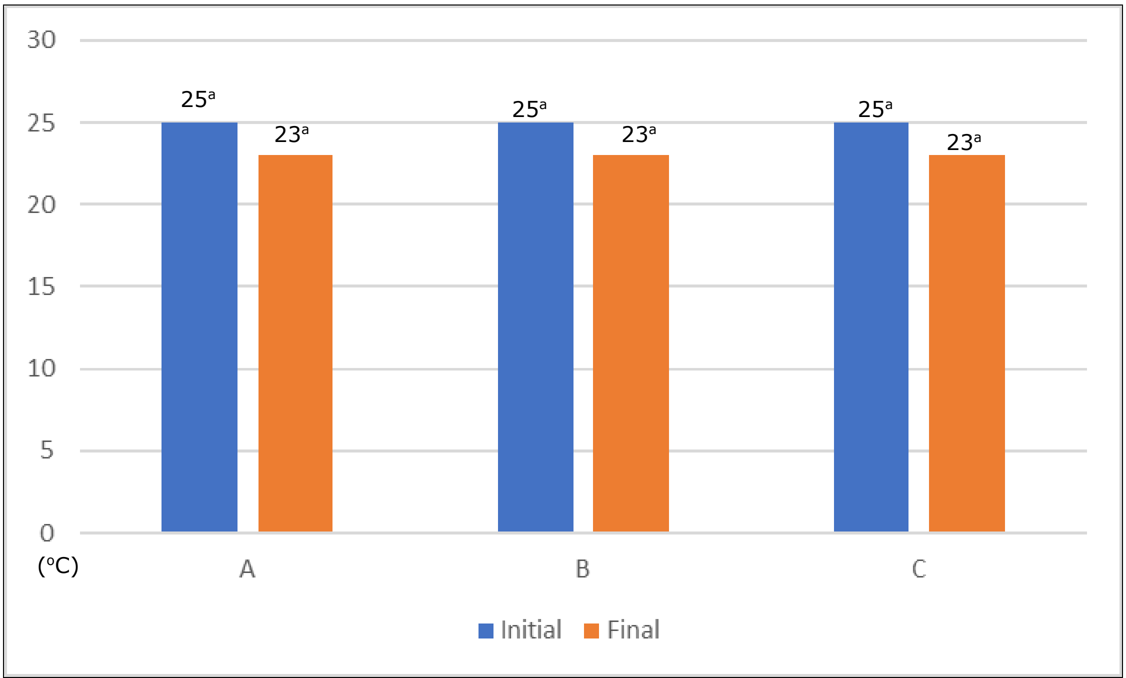

Figure 9. Statistical difference of temperature obtained in the treatment.

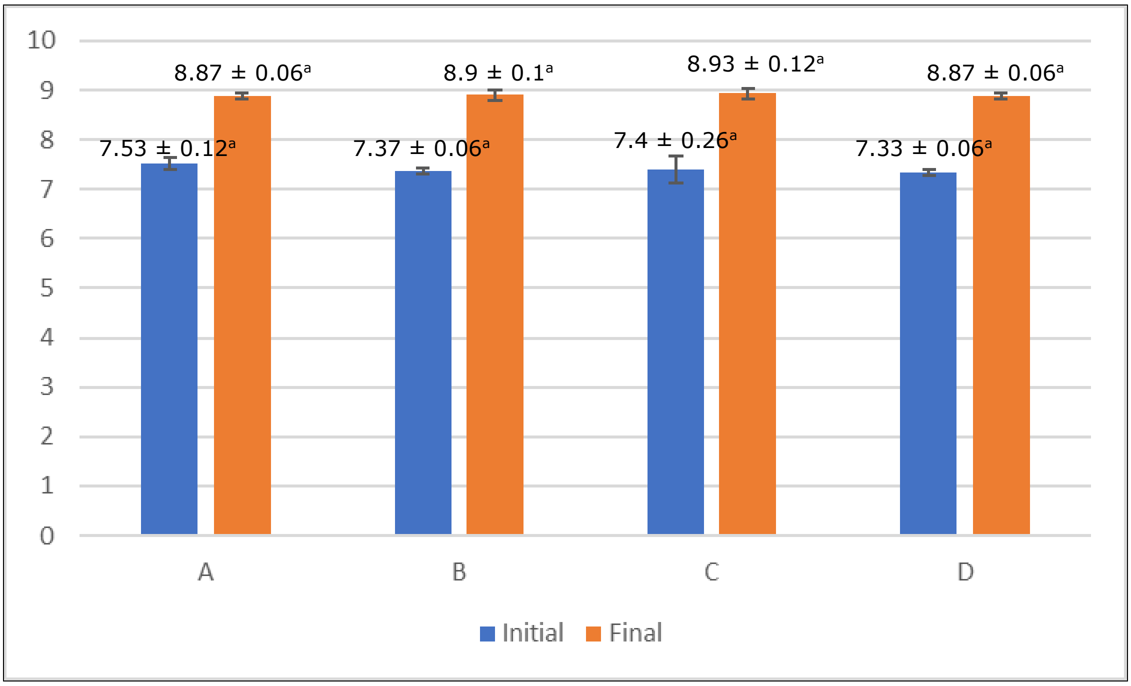

Figure 10. Statistical difference of pH obtained in the treatment.

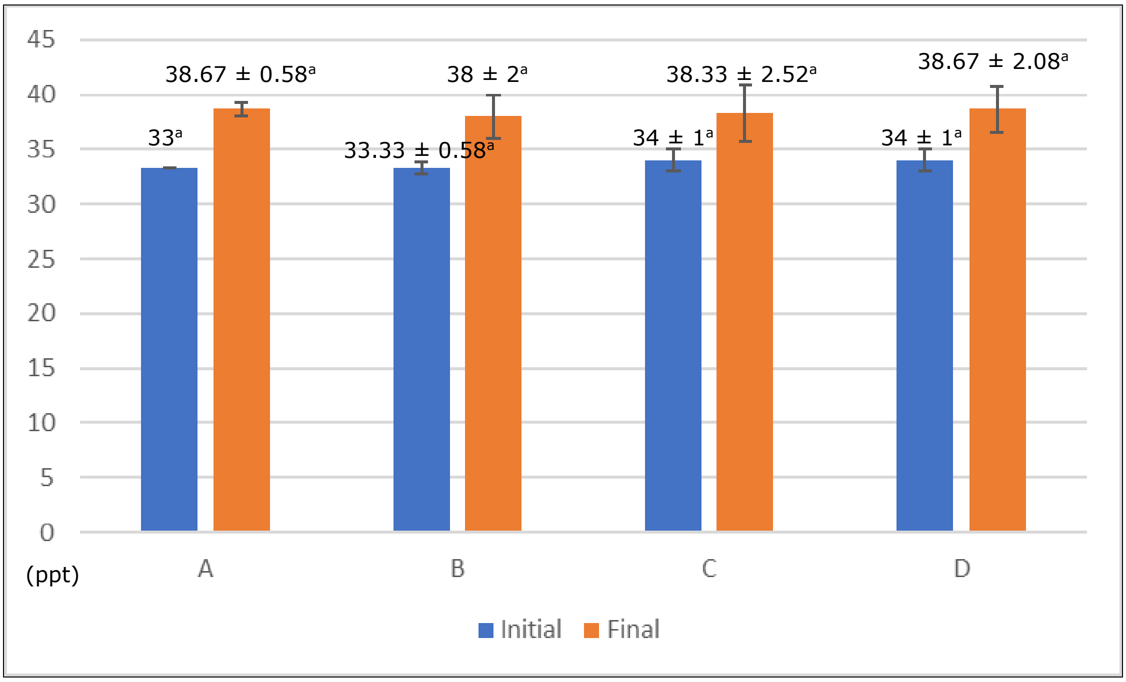

Figure 11. Statistical difference of salinity obtained in the treatment.

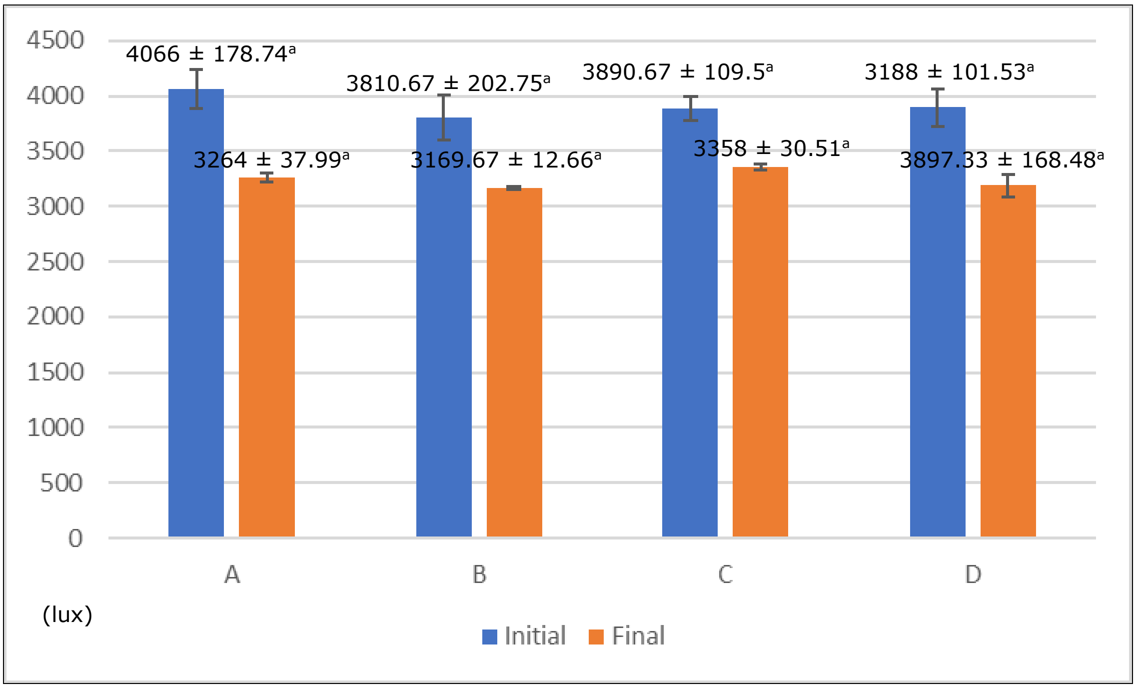

Figure 12. Statistical different of light intensity obtained in the treatment.

Temperature is an essential factor for the life of organisms because temperature significantly affects 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. Being in this temperature range enables the *Spirulina* sp. culture to propagate at its maximum potential (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 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 CO_2 by *Spirulina* sp. as a carbon source for photosynthesis, so that it induced a pH change (Wang et al 2019).

Alkalinity and salinity are also important for microalgae. Salinity played a vital role in the life of aquatic organisms and affected the growth rate because salinity stress would affect osmoregulation (carbohydrate metabolism) through energy consuming processes to overcome high salinity (Çelekli et al 2016). Based on observations in each treatment, salinity in the water medium ranged between 33-38.67 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). Increasing the salinity value affected microalgae. Previous research stated that excessive salinity could cause stress and inhibit the growth of microalgae (Gao et al 2018). Alkalinity can also affect microalgae. Previous research showed that saline stress inhibited photosynthesis and nitrate uptake (Wang et al 2021).

Light is 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 between 3088 and 4298 lux, and the results were still in the field of optimal light intensity for the growth of *Spirulina* sp., which ranged between 1500 and 4500 lux (Richmond 2008).

Conclusions. This study had successfully confirmed the ability of *Spirulin*a sp. to remediate waste water from humpback grouper (*Cromileptes altivelis*). *Spirulina* sp. can reduce nitrate and phosphorus in water up to 89.78% and 84.34% of the waste, following the Indonesian standard. Not only nitrate and phosphate, *Spirulina* sp. could maintain all water quality parameters to follow the standards for fish farming for 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.

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

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