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Growth performance and heavy metal analysis of *Oreochromis niloticus* fed with phycoremediate microalgae supplemented diet formulation

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Abstract

The rising demand for fish as the primary protein source for human consumption is a continuous challenge to meet. Modern-day studies have exploited microalgae as an alternative plant-protein source to replace fishmeal. However, it is critical to protect the fish culture from toxic metal accumulation, which is dangerous to both the organisms and consumers. Hence, this research aimed to study the effects of using microalgae bio-products on the growth performance and heavy metal accumulation in the muscle tissue of *Oreochromis niloticus*. In this study, 2% of *Scenedesmus* sp. cultivated in Bold Basal Medium and wet market wastewater was partially substituted to replace fishmeal. The physicochemical properties of formulated feed were analyzed and compared between control, formulation 1 and formulation 2. The fish were cultured for 12 weeks, and their weight gain was monitored weekly using an analytical balance. The muscle tissue samples were collected and digested using 37% HCl and 65% HNO_3. As a result, the growth performance of the fish in formulations 1 and 2 showed a positive trend and a higher average growth rate of up to 43.38 and 44.85 g, respectively. The effect of seven heavy metals, such as zinc, iron, copper, lead, mercury, manganese, and cadmium, on the fish was analyzed using the Inductive Coupled Plasma Mass Spectrometry (ICPMS) technique. An analysis of variance indicated a significant difference ($p < 0.05$) for zinc, copper, iron, lead, and mercury concentrations but not for manganese and cadmium ($p > 0.05$). It can be deduced that the inclusion of 2% *Scenedesmus* sp. in the diet formulation results in a minor variation in fish growth performance. However, this could contribute to maintaining and improving the growth rate of *O. niloticus* and the concentration of heavy metals in their muscle tissue. Continuous examination of heavy metal accumulation in fish is necessary to ensure the safeness and nutritional quality are adequate for human consumption.

1. Introduction

Malaysia is endowed with flora, fauna, as well as innumerable rivers and lakes. These ecosystems provide sustenance such as protein for their inhabitants. Fish are notoriously known as one of the essential protein sources for Malaysians. It plays numerous vital roles in human health; people who do not get enough vital proteins are susceptible to illnesses (Abdul *et al*., 2015). In addition, Malaysia has considerable aquacultural growth thanks to its vast freshwater bodies. Furthermore, according to Abdul *et al*. (2015), the Malaysian government has recognized aquaculture as a promising arena for investment.

Various challenges, however, have emerged in the aquaculture industry due to the growing use of fishmeal as a primary source of protein in diet formulation, which has contributed to the high cost and limited accessibility of conventional fishmeal. Such issues necessitate the search for new protein sources to fulfil the demand of the aquaculture industry. According to Sirakov *et al*. (2015), microalgae are the most readily available and cost-effective food ingredient. Their appeal is unquestionable due to their great adaptability to harsh settings, high nutritional content, simplicity of cultivation, strong disease resistance, and high growth rates. Moreover, microalgae are cultivated not only as a bio-filter to

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remove nutrients but also as a food supplement (Hattab and Ghaly, 2014), such as *Chlorella* sp. feed for Korean rockfish (Bai *et al*., 2002) and *Scenedesmus* sp. feed for Tilapia (Tartiel *et al*., 2008).

Oreochromis niloticus, popularly known as Nile Tilapia, is a freshwater species that is widely cultivated across the world. According to Wang and Lu (2016), the production of *O. niloticus* has increased over the past decades due to its marketability, suitability, and consistent market pricing. Furthermore, the adaptability of *O. niloticus* to withstand low dissolved oxygen, high ammonia, and high disease resistance has made this species one of the most popular among aquaculture fish farmers However, to ensure that the cultured fish are safe for human consumption, the concentration of metals found in the fish muscle tissues must be monitored periodically.

Metals, which are naturally prevalent in aquatic ecosystems, are a product of human activities (Dusukcan *et al*., 2014). The release of untreated wastewater into the aquatic habitat is a risk element for creatures living there, such as fish. As the concentration of the heavy metal increases, the metals inexorably enter the biogeochemical cycles, posing a threat not only to aquatic organisms but also to consumers.

Phycoremediation denotes the process of removing contaminants, excess nutrients, heavy metals and toxic chemicals from wastewater using micro- or macroalgae. This treatment is not only cost-effective but also ecologically friendly and efficient in removing excess nutrients; nonetheless, the bio-product can be utilized for a variety of purposes, including biodiesel and fish feed (Apandi *et al*., 2018). Moreover, untreated wastewater provides an abundance of nutrients that promote the growth of microalgae. This has allowed microalgae to flourish, resulting in increased nutritional content and, therefore, a reduction in reliance on fishmeal (Mobin and Alam, 2014).

Microalgae received more attention due to their higher protein which is almost like the high-demand fishmeal. Furthermore, compared to animal by-products, microalgae are preferable since animal by-products require the rendering process before being used on the farm (Jedrejek *et al*., 2016). According to Shah *et al*. (2017), the composition of commercial microalgae species such as proximate analysis, amino acids and fatty acids and their mineral content are comparable to the available feed ingredients in the aquafeed industry (Kent *et al*., 2015). *Scenedesmus* sp. are photosynthetic microalgae commonly found in fresh and brackish water. They can adapt to extreme conditions, such as polluted water, making them useful as a pollution indicator (Shubert *et al*., 2014). Besides, *Scenedesmus* sp. is a source of single cell protein up to 40% to 70% by converting solar energy as claimed by Nasseri *et al*. (2011). Lipids are highly significant at various growth stages of many aquaculture animals including fish larvae. In green algae, the average lipid content is 23% while in *Scenedesmus* sp. is 26% (Rakesh *et al*., 2016).

In this study, *Scenedesmus* sp. was used due to its accessibility. It was isolated from Taman Negara Endau-Rompin and cultured. Although *Scenedesmus* sp. is commonly used to treat wastewater (Apandi *et al*., 2018), little is known about the concentration of heavy metals in the fish muscle tissue fed with formulated diets containing *Scenedesmus* sp. Therefore, this study aims to determine the growth performance of *O. niloticus* fed with formulated diets supplemented with *Scenedesmus* sp. as well as the concentrations of heavy metals in the fish muscle tissue.

2. Materials and methods

2.1 Formulation of fish feed

The fish feed was formulated using four main ingredients; fishmeal, soybean meal, wheat flour, and corn grain in specific proportions. All the feed basic ingredients were supplied by Green Aquatic Feed (M) Sdn. Bhd. The formulations adopted from previous research were done by Talip *et al*. (2019) using artificial wastewater, while in this study, real wastewater was used. Besides, the maximum inclusion limit of each basic ingredient used in the formulation was following the Food and Agricultural Organization (FAO) (2014). As shown in Table 1, this experiment consisted of three formulations; a control from pure fishmeal with no microalgae substitution (control), 98.0% pure fishmeal with 2.0% substitution of *Scenedesmus* sp. cultivated in Bold's Basal Medium (F1), and 98.0% pure fishmeal with 2.0% substitution of *Scenedesmus* sp. cultivated in wet market wastewater (F2). In addition, 2% replacement of fishmeal with microalgae was used to ensure that fishes given formulated diets survived throughout the experimental period (Walker and Berlinsky, 2011). The ingredients were weighed using an analytical balance (PB153-L Mettler Toledo, Switzerland) and homogenized in a vertical pulverizer for 15 mins before being molded into 0.25 g spherical pellets. These pellets were then dried in a convection oven at 55°C until the product was reduced to around 65% of its original weight (Sarker *et al*.,2015).

2.2 Physicochemical analysis of formulated fish feed

2.2.1 Diameter of feed

The final weight of the pellet was measured using analytical balance and the diameter was measured using

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Table 1. The composition of ingredients in fish feed formulation using specific protein source based on the maximum inclusion limit by Food and Agricultural Organization (2019).

Ingredients	Composition (%)		
	Control	F1	F2
		98.0% fishmeal with 2.0% of *Scenedesmus* sp. cultivated in BBM	98.0% fishmeal with 2.0% of *Scenedesmus* sp. cultivated in wet market wastewater
Protein supplement			
Soybean meal	19.60	19.60	19.60
Fishmeal	49.00	48.02	48.02
Scenedesmus sp.	-	0.98	0.98
Basal feed			
Corn grain	14.70	14.70	14.70
Wheat flour	14.70	14.70	14.70
Subtotal	98.00	98.00	98.00
Controlled ingredients			
Guar gum	2.00	2.00	2.00
Total	100.00	100.00	100.00

micrometer.

2.2.2 Analysis of hardness

The hardness of the formulated pellets was analyzed using texture analyzer (Stable Micro Systems, United Kingdom). Triplicates of maximum tensile stress were measured with a cylindrical probe with code number of SMS P/36R (Bringas *et al*., 2007).

2.2.3 Moisture content analysis

The analysis of the moisture content of the feed was based on The Association of Official Analytical Chemists (AOAC) Official Method 934. 01 (1990). Approximately 2.0 g of homogenized samples were dried to constant weight at 95°C to 100°C under pressure less than 100 mmHg (Memmert, Germany). The percentage of total solids in the sample was calculated as shown in the formula below (Nielsen, 2014).

$$\% \text{ Moisture} = \frac{\text{Weight of original sample} - \text{Weight of dried sample}}{\text{Weight of original sample}} \times 100$$

2.2.4 Biuret protein analysis

The Biuret crude protein analysis was carried out based on the method described by Patel (2014). 1.0 g of grounded and homogenized fish feed samples were transferred into a 50 mL Erlenmeyer flask. To solubilize the protein, 20 mL of 0.5 M NaOH was added. The mixture was heated for 10 min (Smith, China), filtrated, and the solid particles were removed. The mixture of filtrate and petroleum ether, 15 mL each was centrifuged at 8000 rpm for 5 mins to remove fat. The 30 µL of a clarified aqueous phase and 970 µL of distilled water were pipetted and 4 mL of biuret reagent was added. The mixture was allowed to stand for 30 min. UV-vis spectrophotometer (HACH, USA) was used to obtain the absorbance value at 550 nm for each sample.

2.2.5 Ash and mineral analysis

The feed samples of 2.0 to 10.0 g were reduced to ash in a furnace (Protherm, Turkey) at 550°C and held for 4 h. The weight of the ash was calculated by using Equation 2. The ash was then cooled and 10 mL of 3M HCl was added. The mixture was boiled gently for 10 min, and the solution was filtered into 100 mL volumetric flask. The sample was analyzed by atomic absorption spectrometer (PerkinElmer, USA) and the element content was calculated by using equation 3 below.

$$\% \text{ Ash} = \frac{W3 - W1}{(W2 - W1) \times dry\ matter\ coefficient} \times 100\%$$

Where W1 is the weight of crucible and lid (g), W2 is the weight of crucible lid with sample (g), and W3 is the weight of crucible lid with ash (g).

$$\text{Element content (mg/100 g)} = \frac{C \times D \times 100\ g}{100\ g \times W}$$

Where C is the concentration of mineral in sample (mg/L), D is the dilution factor, and W is weight of sample (g).

2.2.6 Water stability analysis

A time span of 2 hrs was needed to determine the wet durability of the feed in 9 glass beakers that contained 1 g of sample each and 500 mL of tap water. The pH of water was maintained between pH 8 to pH 9 (Chemorpharm, Malaysia). The weight of samples was then measured at intervals of 30 min, 1 hr and 2 hrs after immersion. The undissolved solids were filtered and dried in an oven to achieve accuracy. Equation 4 below was used to calculate the leaching rate of the feed pellet.

$$\text{Leaching rate} = \frac{A \times (1 - r) - R}{A \times (1 - r)}$$

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Where A is weight of pellets before immersion (g), r is moisture content of pellets, and R is the dry weight of the remaining solid (g) (Ighwela *et al*., 2013).

2.3 Culturing Oreochromis niloticus

The experimental setup for culturing the Nile tilapia or *O. niloticus* comprised three 15×30 inches aquariums. A total of twelve *O. niloticus* fingerlings with an average initial weight of 6.50±0.05 g were supplied in each aquarium with replication of tank. The feeding experiment lasted for 12 weeks since *O. niloticus* can attain maturity at the age of 3 months. The first aquarium served as a control with *O. niloticus* not being fed a diet containing microalgae, while the second and third aquariums were fed diets with substitution of microalgae cultivated in Bold's Basal Medium (BBM) and wet market wastewater, respectively. The appropriate amount of feed in the experimental diet was calculated as a percentage of the average weight gain. The amount of feed provided to the fish was modified every 15 days and distributed in three equal parts for 12 consecutive weeks. Each tank received 2.5 watts of aeration from an air pump and 100 L of de-chlorinated tap water for 24 h. They were actively aerated to keep the oxygen saturation at the right level. The water filter in the aquarium was used to eliminate the uneaten leftovers, free-floating particles, potentially harmful chemicals, and fish waste products. All the aquarium tanks were cleaned once a week, with excess feed, faeces, and filthy water being drained out using silicone tubes.

2.3.1 Determination of growth performance of Oreochromis niloticus

All data obtained were computed as described by Bawdy *et al*. (2008). The fish weight gain was calculated using the following formulas:

Weight gain (g/fish) = Mean of weight (g) at the end of experimental period – weight (g) at the beginning of the experimental period.

$$\text{Specific growth rate (SGR)} = \frac{\ln W1 - \ln W0 \times 100}{\text{No. of days}}$$

Where W1 is the final body weight of *O. niloticus* (g), and W0 is the initial weight of *O. niloticus* (g).

Food conversion ration (FCR) = Feed intake (g) / Weight gain (g)

Survival rate (SR) = No. of fish × 100 / No. of fish introduce

2.4 Water quality analysis

2.4.1 Determination of dissolved oxygen concentration

The level of dissolved oxygen in each experimental tank was determined using a dissolved oxygen meter (Amtast, China).

2.4.2 Determination of ammonia concentration

The determination of ammonia level in the water sample was determined using a standard method of nitrogen ammonia analysis 8038 (HACH, 1979). A 25 mL sample cell was filled with water sample to the mark and another 25 mL sample cell was filled with demineralized water. Three drops of mineral stabilizer were added to both sample cells and inverted several times to mix. Both sample cells were then added with 3 drops of polyvinyl alcohol dispersing agent and inverted several times until fully dissolved. Nessler reagent of 1.0 mL was pipetted to each sample cell and inverted several times. After 1 min, the blank sample cell was placed in the cell holder followed by the cell with water sample. The reading was recorded and analyzed using one-way ANOVA.

2.4.3 Determination of nitrite concentration

The nitrite level in the water sample from each formulation tank was determined using a powder pillow of Diazotization method 8507 (HACH, 1979). A 10 mL cell was filled to the line with a water sample. The content of One NitriVer 3 Nitrite Reagent Powder Pillow was added to the water sample and inverted several times to mix. At this point, the pink colour appeared as nitrogen was present in the water sample. After 10 mins, the cell was placed in the cell holder and the reading of the nitrite level in the water sample was recorded.

2.4.4 Determination of nitrate concentration

Determination of nitrate level in the water sample from each formulation tank was determined using reduction method 8192 (HACH, 1979). A 50 mL graduated mixing cylinder was filled with 30 mL of the water sample. The contents of One NitraVer 6 Nitrate Reagent Powder Pillow were added and the cylinder was shaken continuously for 3 min. The cadmium in the One NitraVer 6 Nitrate reagent was allowed to settle for 2 min. Then, 10 mL of the prepared sample was transferred into a 10 mL cell sample. The content of NitriVer3 Nitrite Reagent was added to the 10 mL cell sample and shaken to dissolve. A pink colour appeared as there was the presence of nitrate in the water sample. After 10 min, the 10 mL cell sample was placed in the cell holder and the reading was recorded.

2.5 Biological fish sampling

A set of dissecting kits was used to collect muscle tissue of *O. niloticus*. All glassware was kept in 10% Nitric acid (HNO_3) overnight before being rinsed with

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distilled water. The skin was meticulously removed with a pair of tweezers and a scalpel. To extract the most flesh, the muscle tissue samples were collected along the lateral line towards the tail. The muscle tissue was carefully removed to ensure that there was no skin or scales in the sample. Subsequently, 50.0 g of *O. niloticus* muscle tissue samples were collected from each formulation and replicated for further analysis.

2.5.1 Heavy metals analysis

In this experiment, muscle tissue or known as fish flesh was chosen to ensure the fish given a formulated diet would not have harmful effects and contribute to the accumulation of heavy metals. One gram of *O. niloticus* muscle tissue was weighed in a 250 mL beaker for the digestion process. In the beaker, a 3:1 combination of HCl and HNO_3 in a volume of 10 mL (volume-to-volume) was added. The beaker containing the mixture of digesting solution and weighed sample was then placed on a hot plate in a fume cupboard at 100°C for 2 hrs. Following the cooling process, 20 mL of the digesting solution was added to the beaker (Abiona *et al*., 2018). The mixture was filtered into a 250 mL Buchner borosilicate filtering flask, and the filtrate was supplemented with ultra-pure water to the mark. The procedure was done three times for each experimental tank, and the samples were analyzed using Inductive Coupled Plasma Mass Spectrometry (ICPMS) (PerkinElmer SCIEX, USA).

2.6 Statistical analysis

The data for physicochemical analysis of the formulated fish feed, growth performance of *O. niloticus*, water quality conditions, and heavy metals analysis were analyzed using SPSS software. All the results of the analysis obtained were tested using one-way ANOVA and expressed as mean±standard deviation.

3. Results and discussion

3.1 Physicochemical analysis of formulated fish feed

Oreochromis niloticus is known as the second most popular fish species cultured worldwide. To ensure a high quality of fish produced, a good production pellet used to feed the organism is one of the most important factors to analyze. This section discussed the physicochemical properties of the feed pellet including weight, diameter, hardness, moisture content and biuret total protein (Table 2). *Oreochromis niloticus* is categorized into a few life stages, which are fry, fingerlings and adult. Different life stages require different diameters and weights of the pellet. The average pellet diameters for all formulations were 5.105±0.078 mm, 5.064±0.053mm and 5.063±0.047 mm as respective to control, F1 and F2 while the weight of the pellet was 0.156±0.007 g, 0.164±0.022 and 0.167±0.019 for diet control, F1 and F2 respectively. According to FAO (2014), the fry stage of *O. niloticus* requires between 1 to 3 g with 1 to 2 mm diameter of the feed pellet for the fish feeding while the juvenile life stage is between 3 to 30 g with 2 to 3 mm in diameter. Adult *O. niloticus* is further classified as having body weight between 30 to 100 g, 100 to 250 g and more than 250 g with a diameter of feed pellet 2 mm, 3 mm and 4 mm respectively to enhance the feeding efficiency.

According to Lee *et al*. (2016), the pellet size of the feed would affect the growth performance of fish. It is important to ensure a suitable type and size of the pellet to stimulate the maximum growth performance of the fish as it will affect the amount of feed of individual fish that can be ingested over a period. Moreover, a wrongly chosen size of pellets and a high number of pellets may affect the water quality and cause feed wastage as fish are unable to ingest the required number of feeds A study by Azaza *et al*. (2010) found that smaller feed particles have a high surface-to-volume ratio and are digested faster than larger feed particles. Moreover, the smaller size of the feed particle will be evacuated more rapidly and enter the intestine without enough gastric digestion, thus reducing the nutrient absorption efficiency compared to the larger size particle. The average diameter value of formulated feed in this study exceeded the advised value by FAO (2014). A study by Lee *et al*. (2016) recommended selecting the largest size of the feed. This will encourage the fish to eat, compared to smaller feed where the fish used more energy in finding and eating more pellets. This will result in inefficient feeding. The hardness of fish pellets is commonly

Table 2. Physicochemical properties of formulated fish feed for *O. niloticus*

Parameter	Formulated Fish Feed (mean±standard deviation)		
	Control	F1	F2
		98.0% fishmeal + 2.0% *Scenedesmus* sp. cultivated in Bold's Basal Medium	98.0% fishmeal + 2.0% *Scenedesmus* sp. cultivated in wet market wastewater
Weight (g)	0.156±0.007	0.164±0.022	0.167±0.019
Diameter (mm)	5.105±0.078	5.064±0.053	5.063±0.047
Hardness (N)	13.382±0.935	14.739±0.652	15.379±0.069
Moisture content (%)	12.030±0.170	20.300±0.080	11.590±0.050
Biuret total protein (%)	14.000±0.640	24.910±0.520	64.370±0.940

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derived from a low moisture content of the pellet, which might reduce the feeding efficiency of the fish. Pellet hardness for all diet formulations increased from 13.382±0.935 N for control to 14.739±0.652 N and 15.379±0.069 N for F1 and F2 respectively (Table 2). This might be due to the weight of the fish pellet could not be controlled before and after drying until constant weight. Results have revealed that the hardness values of all control, F1 and F2 are significantly different ($p< 0.05$).

The moisture content parameter of the pellet also affects its preference and acceptability by the fish as softer pellets are usually preferred compared to harder pellets (Aas *et al*., 2011). According to Dragonovic *et al*. (2011), the optimum hardness value following the commercial guidelines in making fish feed is between 740 kg and 850 kg to ensure the physical integrity of the pellets produced indirectly easing the handling and transportation process. Other than that, the hardness of the feed pellet is also related to its water stability which will determine the nutrient retention capacity and sinking velocity of the pellet (Obirikorang *et al*., 2015). Fish pellet that comes with different ingredients will inherit different binding properties leading to different stability and quality of the pellet. The moisture content of F2 showed the lowest average moisture value which was 11.59±0.05 % compared to control and F1 (Table 2). However, it is still within the optimum range (below 11%) to ensure a longer shelf life (Oehme *et al*.,2012). Apart from that, differences in the data on the moisture content of the pellet samples tested might be due to the failure to maintain a constant weight for each pellet sample before and after the pelletizing and drying processes. The findings have shown that all three diet formulations were significantly different ($p<0.05$).

The diet formulation containing 2% microalgae substitution cultivated in wet market wastewater, showed the highest biuret total protein content, which was 64.37±0.94 % compared to the diet in control and F1. This finding was in agreement with a study by Duncan and Klesius (1996) where the researcher found that *Spirulina* sp. was a good source of protein for animal feed. It also gives a positive increment in the growth performance of the fish cultured. The highest hardness of the formulated feed found was F2 which was 15.379±0.069 N. The main ingredients used that might have contributed to the pellet's quality and hardness were wheat flour, guar gum, and *Scenedesmus* sp. The optimum moisture content found in this study was from diet F1 with 20.30±0.08%, while diet F2 had optimum biuret total protein content with the percentage of total protein 64.37±0.94%. It is important to have an optimum moisture and protein content as the higher moisture content will reduce the hardness of the feed and lead to an increased feeding efficiency of the fish.

3.2 Water stability in fish pellet

The addition of microalgae *Scenedesmus* sp. in the diet formulation to replace fishmeal of the feed had resulted in the reduction of water stability of the feed pellet from 30 mins of the stability testing to 2 hrs (Table 3). This might be caused by the usage of guar gum as a binder in the diet formulation for the fish. Moreover, microalgae such as *Scenedesmus* sp. used is also categorized as a source of non-starch polysaccharides (NSP). NSP contains anti-nutritive effects which might have resulted in growth reduction, lowered interaction between nutrient and enzyme and slowed glucose intake by the fish (Sinha *et al*., 2011). This study was contrary by Paolucci *et al*. (2015) where the addition of microalgae *Scenedesmus* sp. in the diet formulation of *O. niloticus* as a protein replacer had encouraged their potential contributor as a binding agent. The biopolymer found on the cell wall of the microalgae could function as the binding agent for the pellet. In fact, these findings were in agreement with previous research done by Allen (2016), where the addition of *Spirulina* as a protein replacer had decreased the water stability of feed pellet. Thus, replacement of fishmeal with microalgae could reduce the stability of pellet which will also affect the feeding efficiency of the fish.

3.3 Ash and mineral analysis of fish pellet

This study observed the present of minerals including Calcium (Ca), Potassium (K), Magnesium

Table 3. Water stability of feed pellet from three different diet formulations.

Diet	Average water stability of formulated fish feed, % (mean±standard deviation)		
	30 mins stability	1 hr stability	2 hr stability
Control	95.286±0.522	92.404±0.667	88.424±0.628
F1 98.0% fishmeal + 2.0% *Scenedesmus* sp. cultivated in Bold's Basal Medium	90.791±0.806	60.392±0.639	57.504±0.172
F2 98.0% fishmeal + 2.0% *Scenedesmus* sp.	91.511±0.229	89.226±0.524	84.813±0.587

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(Mg), Sodium (Na), Iron (Fe) and Zinc (Zn) as outlined by National Research Council (1993) (Table 4). The average value of all minerals in all diet formulations were under recommended limits except for Na mineral in F2. This might be due to the incomplete diet supplements such as mineral premix in the formulation. In feed industry, mineral premix is added into feed formulation to fulfil the dietary mineral requirement of the fishes. However, this study lacks mineral premix supplements resulting in the low level of minerals in the fish diet. Nonetheless, comparing the mineral compositions between all formulations, F2 showed an improvement among all with addition of 2% *Scenedesmus* sp. cultivated in wet market wastewater. The data analysis has showed that mineral compositions of all diet formulations were significantly different ($p<0.05$). The improvement in average mineral composition value in diet F2 in might be due to the absorption of excess nutrients by the microalgae during the cultivation period through the phycoremediation process of wet market wastewater (Table 4).. Besides, it was also necessary to have micro minerals supplementation since the concentration of these minerals is naturally low in feed ingredients (Prabhu, 2015). It can be deduced that the addition of phycoremediate microalgae in F2 results in optimum mineral composition.

3.4 Growth performance of Oreochromis niloticus fed with formulated diets

Figure 1 depicts the growth performance of *O. niloticus* fingerlings in terms of weight gain (mean ± standard deviation) (g) from week 1 (W1) to week 12 (W12). The growth performance of *O. niloticus* in all formulations improved from W1 to W12, notably in F1 and F2 when compared to the control. These findings were observed due to the inclusion of plant protein sources in diet formulations, which aided in the development of the fish's metabolism. The findings were consistent with Patnaik *et al*. (2019), who reported replacing conventional fishmeal with 50% *Scenedesmus obliquus* increased fish weight. The fish weight gain can be detected as early as W2, when fish from F2, showed the greatest weight gain compared to those from the control and F1. From W2 to W6, a similar growth pattern can be noticed, with fish from F2 showing the significant growth performance when compared to other formulations.

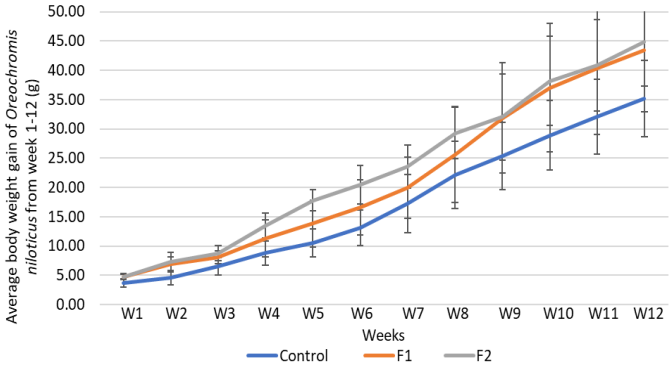

Figure 1. Growth performance of *O. niloticus* cultivated in three separate aquariums fed with three different formulations for 12 weeks.

On W9, *O. niloticus* from F1 showed an increasing growth performance as weight gained compared to the fish from F2. This finding was similar to a study by Santos *et al*. (2019), in which the addition of microalgae *Schizochytrium* sp. in feed increases the growth performance of *O. niloticus* linearly, with improvement percentages for final weight gain of 37.50% and 47.67%, respectively. Moreover, Saiyasaeng *et al*. (2014) observed a greater improvement of 42.46% final weight gain and 82.69% weight gain of *O. niloticus* with 7.5% of microalgae substitution in *O. niloticus* fish diet. A favorable increment in *O. niloticus* growth performance might be attributed to the association of the protein content of *Scenedesmus* sp. itself. According to Becker (2007), the protein, lipid, and carbohydrate percentage concentrations in microalgae *Scenedesmus* sp. are ranging from 50 to 56%, 12 to 14%, and 10 to 52%, respectively. However, no significant differences ($p >$

Table 4. Mineral composition of different diet formulations for *O. niloticus*

Mineral composition (mg/ g)	Diet formulation (mean±standard deviation)				
	Control	F1 98.0% fishmeal + 2.0% *Scenedesmus* sp. cultivated in Bold's	F2 98.0% fishmeal + 2.0% *Scenedesmus* sp. cultivated in wet	Dietary requirement	References
Na	0.2100±0.0020	1.2170±0.0170	1.4310±0.0250	1.50	Shiau and Lu (2004)
K	0.0820±0.0010	2.7020±0.0340	3.6730±0.0210	2.00 - 3.00	Mjoun *et al*. (2010)
Mg	0.0350±0.0011	0.2970±0.0160	0.2970±0.0390	0.60	NRC (1993
Ca	1.4470±0.0120	2.1130±0.0060	2.2490±0.0600	7.00 - 7.50	Mjoun *et al*. (2010)
Fe	0.0020±0.0001	0.0110±0.0001	0.012±0.00006	1.20	El-Serafy *et al*. (2007)
Zn	0.036±0.001	0.026±0.006	0.01 0± 0.0001	0.03	Mjoun *et al*. (2010)

*Na: Sodium, K: Potassium, Mg: Magnesium, Ca: Calcium, Fe: Iron, Zn: Zinc

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0.05) across all formulations from W7 to W12. *Oreochromis niloticus* from F2 had the highest weight gain throughout the experimental period, with an average weight gain of 44.85±7.51 g, compared to *O. niloticus* from the control and F1, which had final average body weights of 35.21±6.49 and 43.38±10.42 g, respectively. Moreover, a study on the ingestion rate of fish fed with different types of diets conducted by Li *et al*. (2015) has revealed that the addition of the prototype developed in the diet ingredients of the fish boosted the ingestion rate of the juvenile fish, therefore enhancing the fish growth.

Following completion of the experimental diet, the nutritional indices of *O. niloticus* were also examined. Table 5 shows the nutritional indices of *O. niloticus* at the end of the cultivation period. *O. niloticus* from the control, which was fed with formulated diet without microalgae substitution, had an SGR value of 2.05%, whereas fish from F1 and F2, had increasing percentages of SGR, which were 2.14 and 2.19% respectively. Besides, the FCR value of the fish cultured in the control falls from 2.82 to 1.97 for F1 and 1.89 for F2. The high energy composition of feed results in a lower FCR value and higher nutrient retention. These results indicated that the addition of *Scenedesmus* sp. in the diets enhanced the FCR value of the feed. This finding was corroborated by Patnaik *et al*. (2019), who found that a diet formulation containing 50% *Scenedesmus obliquus* as a fishmeal replacer had the lowest FCR value. Nonetheless, *O. niloticus* from the control showed a lower survival rate of 58.3%, with five dead fish compared to F1 and F2, which had a comparable survival rate of 66. 67% with four dead fish. It can be deduced that the addition of phycoremediate microalgae to diet formulation boosted fish growth performance, resulting in an optimum average weight gain of *O. niloticus* in F2 of 44.85 g compared to the F1 and control.

3.5 Water quality in culturing Oreochromis niloticus

There were 4 water quality parameters analyzed every 7 days within 12 weeks which were dissolved oxygen (mg/L), Ammonia (mg/L), Nitrite (mg/L) and Nitrate (mg/L). Figure 2 shows a graph of water quality analyzed from W1 until W12 for 3 different formulations.

The average dissolved oxygen concentration of control, F1, F2 were 5.22 mg/L, 5.27 mg/L and 5.24 mg/ L, respectively. A slightly decreasing value of dissolved oxygen in F2 might be caused by the increasing uptake by microorganisms during the breakdown of accumulated organic matter in the tank. According to Caldini *et al*. (2015), the optimum range of DO levels in aquaculture specific for *O. niloticus* must be at least more than 3.0 mg/L. In contrast, Ross (2002) reported that on the lower limit, the concentration of DO level of 3.0 mg/L should be the minimum for the optimum growth of *O. niloticus*.. A study by Setiadi *et al*. (2018) on the water quality analysis and survival of Red Tilapia in an aquaponic system, the average concentration of DO levels in all experimental tanks was at the lowest optimum concentration around 3.0 mg/L to 3.5 mg/L. There was no significant difference ($p > 0.05$) of the concentration of dissolved oxygen for F1, F2 and control since the dissolved oxygen is more than 5.0 mg/L and all the fishes from all formulations survived.

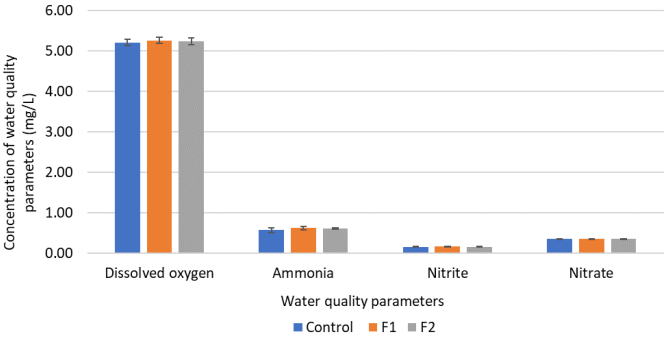

Figure 2. Water quality parameters of three different formulations.

Ammonia is the principal nitrogenous waste produced by aquatic animals via metabolism and excreted through the gills. In this study, analysis of variance (ANOVA) showed a significant different concentration of ammonia in F1, F2 and control. Throughout 12 weeks of the experimental period, F1 showed the highest average concentration of ammonia which was 0.61 mg/L, while the lowest average concentration of ammonia was control with the concentration of 0.57 mg/L. According to Nyanti *et al*. (2012), the concentration of ammonia is commonly high at fish culture site due to waste such as faeces release by the fish itself into the water. In addition, the optimum ammonia concentration in water quality specific for *O. niloticus* culture is between 0.17 – 3.87 mg/L (Caldini *et al*., 2015). On the other hand, Celik (2012) found that, the concentrations of ammonia ranging from 0.9 mg/ L – 1.9 mg/L are considered as good and desirable for *O. niloticus* culture. The concentration of ammonia increasing in F1 and F2 in this study might be due to the accumulation of uneaten feed and fish faeces. However, the average concentration of ammonia in the F1, F2 and control are still under the optimum range.

The control and F1 shared the highest average concentration of nitrite which was 0.16 mg/L while the average nitrite concentration for F2 was 0.15 mg/L. All the control, F1 and F2 showed a significant average nitrite concentration and considered acceptable as the nitrite reading is under the optimum value for *O. niloticus* culture. A study by Gorlach-Lira *et al*. (2013)

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reported that, the concentration of nitrite in water during the experimental period was ranged from 0.001 to 0.28 mg/L in the cage culture system and the value reported for the study was in line with the optimum concentration of nitrite which is just less than 0.3 mg/L (Boyd, 1998).

Besides, the average concentration of nitrate in the F1, F2 and control, throughout 12 weeks were not significantly different ($p > 0.05$). The highest average nitrate concentration was 0.352 mg/L from F1, followed by control with 0.351 mg/L, while F2 showed a slightly lower average concentration of nitrate 0.349 mg/L. According to Boyd (1998), the desired nitrate concentration in water for aquaculture was 0.2 to 10 mg/L. In contrast, Caldini *et al*. (2015) reported that the optimum concentration of nitrate was between 0.2 to 219 mg/L. Environmental Protection Agency (EPA) set the maximum contaminant level for the concentration of nitrate for drinking water was 10 mg/L. These results proved that the nitrate concentration in all formulations was under the optimum range. It can be concluded that the concentration of dissolved oxygen, ammonia, nitrite, and nitrate in the water of all formulation tanks were within the range.

3.6 Concentrations of heavy metals in Oreochromis niloticus muscle tissue

A total of three *O. niloticus* individuals were collected from each formulation to analyze heavy metals in their muscle tissue. Analysis of variance (ANOVA) revealed a significant difference in zinc (Zn), copper (Cu), iron (Fe), lead (Pb), and mercury (Hg) concentrations between three formulations ($p < 0.05$), whereas the concentrations of both manganese (Mn) and cadmium (Cd) in *O. niloticus* muscle tissue from three formulations were similar ($p > 0.05$) (Figure 3). The concentration of Zn in *O. niloticus* muscle tissue from F2 indicated 0.370 mg/kg, while the concentrations in the control and F1 were lower (0.310 and 0.347 mg/kg, respectively). According to Li and Huang (2016), the weight gain of *O. niloticus* would rise linearly as the amount of zinc supplemented in the meal increases. The higher concentration of Zn may alter the secondary structure of the protein by reducing the α-helix and increasing the β-sheet composition of gill tissue. Moreover, the accumulated Zn in fish is proportional to the Zn concentration in the diet. Thus, it is harmful to both fish and humans. According to the Malaysian Food Act and Regulation (1985), the permitted limit for maximum concentration of Zn is 100 mg/kg, althoughthe Food and Agricultural Organization (FAO) encouraged 40 mg/kg. This investigation indicated that the concentration of Zn in fish muscle tissue from all experimental tanks was within the acceptable limits set by the organizations.

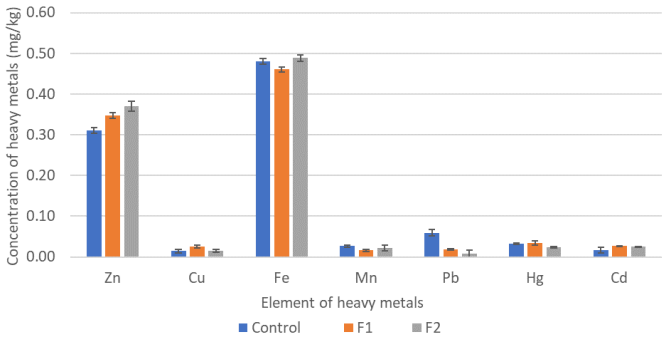

Figure 3. Heavy metal concentrations in *O. niloticus* muscle tissue fed with three diet formulations.

In the marine ecosystem, fish may directly absorb Cu from the water column and their diets; however, the concentration of Cu absorbed by freshwater fish in the ecosystem is still insufficient to meet their requirements (Zhang and Nakajima, 2014). In this study, the average concentration of Cu in *O. niloticus* muscle tissue from the control, F1 and F2 was observed at 0.014, 0.026 and 0.015 mg/kg, respectively. According to both regulation (FAO and MFAR), the maximum allowable limit concentration of Cu in fish muscle tissue was 30 mg/kg. This demonstrated that the concentration of this heavy metal elements in the *O. niloticus* muscle tissue did not surpass the values recommended by both national and international organizations. The average Fe concentration in F2 was 0.49 mg/kg, followed by the average Fe concentration in the control, which was 0.48 mg/kg. Meanwhile, F1 showed lower average Fe concentration of 0.46 mg/kg. The MFAR and FAO have outlined that the maximum permitted limit of Fe in fish muscle tissue is 30 mg/kg. According to Ng and Romano (2013), the optimal Fe concentration for *O. niloticus* is from 150 to 160 mg/kg, which contradicts to Kasozi *et al*. (2019), who claimed that the concentration of Fe ranged were ranging from 30 to 170 mg/kg. This study has shown that the concentration of Mn in the control was the highest (0.03 mg/kg), whereas the concentration of Mn in F1 was the lowest. Both regulations (FAO and MFAR) did not recommend a maximum concentration of Mn since the value was reported as unknown.

Lead, or Pb was found in the greatest quantity in fish from the control, which was 0.06 mg/kg, while the lowest was found in fish from F2 at 0.01 mg/kg. The average concentration of Pb in F1 was slightly higher than in F2 by 0.02 mg/kg. MFAR has recommended the maximum allowable limit concentration of Pb in fish muscle tissue is 2 mg/kg, meanwhile the FAO recommended 0.5 mg/kg. These results showed that the concentration of Pb metal in fish from all formulations is within the acceptable ranges specified by both MFAR and FAO. The results for mercury levels in *O. niloticus*

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muscle tissue in all formulations were below the FAO and MFAR permissible limits. F1 had the highest average concentration of Hg with 0.034 mg/kg, while the control had a slightly lower reading of Hg with 0.032 mg/kg. *Oreochromis niloticus* in F2 fed with a formulated feed supplemented with wastewater microalgae, resulting in the lowest Hg average concentration of 0.024 mg/kg. According to the MFAR and FAO the maximum permitted concentration of Hg in fish muscle tissue is 0.5 mg/kg. These findings have revealed that the diet formulation of *O. niloticus* with microalgae substitution had no effect on the concentration of Hg in muscle tissue of the fish culture. Cadmium is regarded as one of the unessential heavy metals in aquaculture. In this study, F1 and F2 shared the highest average concentration of Cd in *O. niloticus* muscle tissue, which was 0.03 mg/kg, while the control had the lowest with only 0.01 mg/kg. According to the MFAR, Malaysian maximum permitted concentration of Cd in fish muscle tissue is 1 mg/kg, while referring to FAO, maximum upper limit concentration of Cd in fish muscle tissues is 0.5 mg/kg.

4. Conclusion

The replacement of fishmeal as the main protein source with 2% of *Scenedesmus* sp. improves the growth performance of *O. niloticus* in F2 when compared to the control and F1. Replacement of fishmeal with *Scenedesmus* sp. cultivated in BBM and wet market wastewater as an alternative plant- protein source does not lead to heavy metals accumulation. This was proved with the concentrations of heavy metals identified in the muscle tissue of *O. niloticus* were within the permissible limits by both Food and Agricultural Organization and the Malaysian Food Act and Regulation.

Conflict of interest

The authors declare no conflict of interest.

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