COMPARATIVE STUDY ON THE GROWTH, NUTRITIONAL COMPOSITION, PIGMENT AND HEMATO-BIOCHEMICAL INDEX OF NILE TILAPIA (*Oreochromis niloticus*) FRY FED WITH SELECTED MICROALGAE

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Roll No.: 0120/02

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Session: 2020-2021

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Aquaculture

Department of Aquaculture
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JUNE 2021

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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List of Abbreviation

Acronym	Definition
∑ PUFA	Total Polyunsaturated Fatty Acid
∑MUFA	Total Monounsaturated Fatty Acid
∑SFA	Total Saturated Fatty Acid
µg/g	Microgram/gram
AA	Arachidonic Acid
ANOVA	Analysis of Variance
BUN	Blood Urea Nitrogen
CFU	Colony Forming Unit
DHA	Docosahexaenoic Acid
DO	Dissolve Oxygen
EPA	Eicosapentaenoic Acid
FAME	Fatty Acid Methyl Esters
FCR	Feed Conversion Ratio
g/dl	gram/deciliter
GCMS	Gas Chromatography and Mass Spectrophotometry
Hb	Hemoglobin
Hct	Hematocrit
HUFA	Highly Unsaturated Fatty Acid
K	Condition Factor
LA	Linoleic Acid
LYM	Lymphocytes
mg/dl	Milligram/Liter
NO_2-N	Nitrite Nitrogen
PLT	Platelet
RBC	Red blood cell
SE	Standard Error
SGR	Specific Growth Rate
SRP	Soluble Reactive Phosphorous
TAN	Total Ammonia Nitrogen
WBC	White Blood Cells

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Abstract

An 8-week feeding trail was conducted to evaluate the effects of two selected marine microalgae strain *Nannochloropsis* sp. and *Tetraselmis* sp. on growth performance, water quality, hemato-biochemical index, carotenoid concentration, nutrition and biochemical composition of Nile tilapia *Oreochromis niloticus* fry (initial weight 0.023±0.0001 g, mean ± SE). Treatments were designed with five experimental diets: CF (0% microalgae which is control feed), N25 (25% *Nannochloropsis* sp.), T25 (25% *Tetraselmis* sp.), N50 (50% *Nannochloropsis* sp.) and T50 (50% *Tetraselmis* sp.) respectively. Two hundred seventy Nile tilapia fry were randomly assigned in 15 rectangular experimental tanks in triplicate form of each treatment. To find the data of growth performance initial and final body weight was recorded. To check the survivality initial and final number of fish was counted. Physical parameters of water quality were checked daily and chemical analysis of water quality parameters were done weekly. At the end of 8-week experiment, fish were sampled for determination of total carotenoid concentration, hemato-biochemical parameters, proximate composition and fatty acid profile of whole Nile tilapia fish tissue and significant ($p < 0.05$) changes of values were observed. The results revealed that supplemented microalgae alone could not significantly enhanced the growth performance than control ($p < 0.05$). Low palatability and antinutritional factors may be responsible. Significant high survival, better TAN, NO_2-N and SRP was noticed in T25 than control ($p < 0.05$). The hemato-biochemical parameters exhibited significant increase of certain parameters within reference range, however significant decrease in some blood parameters was noticed also ($p < 0.05$). Although higher percentage of microalgae in diet has reduced the protein concentration in whole fish muscle compared to control but high lipid and carbohydrate was recorded in N50 and T50 treatment respectively. Comparatively higher carotenoid concentration was revealed in N50 than control. Increasing dietary *Nannochloropsis* sp. and *Tetraselmis* sp. has also significantly ($p < 0.05$) increased the percentage of saturated fatty acids (SAFs), polyunsaturated fatty acids (C20:5n-3, C22:6n-3 etc.) compared to control. Thus, this study provides significant information about beneficial impacts of *Nannochloropsis* sp. and *Tetraselmis* sp. on Nile tilapia fish.

Keywords: Nile tilapia, microalgae**,** growth performance, water quality, hemato-biochemical parameters, carotenoid, nutrition, fatty acid

Chapter-1: Introduction

Microalgae are a diverse group of aquatic organisms that can be found in both marine and freshwater environments. They are unicellular, photosynthetic organisms which can be converted into new algal biomass with the help of light, nutrient, water and carbon dioxide. In aquaculture, they are considered as one of the most prominent aquafeeds or feed supplement as they have high productivity, have access to absorb carbon-di-oxide from the atmosphere, can grow on any environmental condition without being damaged and their suitability for sustainable mass cultivation (Draaisma et al., 2013).

Most recently the term biofuel production from microalgae has grabbed the world's attention, therefore this technology possesses very strongly positive impression in mercantile approaches (Brennan and Owende, 2010). Besides, there are many uses of molecules from these phototrophic micro-organisms in human and animal food, health, and cosmetology. Fishmeal is very common ingredient of feed for fish. About 3724 thousand tones fishmeal is used in 2006 in aquaculture industry (Tacon and Merian, 2008). Now the increasing evident is showing that such continued consumption of this natural resource will unsustainable both environmentally and economically. Thus, an alternative feed ingredient must be used to supply complete nutritional value. In these aspect microalgae as aquafeed has gained popularity day by day due to their appropriate size, high nutritional value such as a good source of polyunsaturated fatty acid (PUFA) like eicosapentaenoic acid-EPA, arachidonic acid-AA, linoleic acid-LA and docosahexanoic acid-DHA, high quality proteins and sterols (Muller-Feuga, 2000; Hemaiswarya et al., 2010), disease resistant power (Spolaore et al., 2006). also because of their antioxidant content (Hemaiswarya et al., 2010), which are essential for larval development, reduce mortality, faster larvae growth and transformation index (Muller-Feuga, 2000; Hemaiswarya et al., 2010).

The aquaculture is a rapid growing sector and continuously increasing its production. The selection of microalgae for culture must be done on the basis of a good nutrient composition and an absence of toxins that might be transferred into the food chain (Velichkova et al., 2012). The main purpose of microalgae culture is to feed for potential aquatic animals. Currently 30% of the world algal production is being used for animal feed (Sharma et al., 2013) but the use in aquaculture is mainly for larval fish, mollusks, and crustaceans (Hattab et al., 2014). Other than that, microalgae also contain

numerous bioactive compounds such as pigment components which are responsible and main source for the coloration of different fish species (Becker, 2013).

In this study, *Nannochloropsis* sp. and *Tetraselmis* sp. was used. *Nannochloropsis* sp. are unicellular small green algae with polysaccharide cell wall containing only one chlorophyll, namely chlorophyll a and also a good source of protein, vitamins and bioactive compounds. This alga has the potentiality to enhance the nutritional quality of the human diet through enriching the n-3 fatty acids of the fish flesh (Gbadamosi and Lupatsch, 2018). *Tetraselmis* sp. is large green flagellates widely used for feeding juveniles, bivalve molluscs, penaeid shrimp larvae and rotifers. This genus has rapid growth rate, good sources of vitamin E, large spectrum of antimicrobial activity and probiotic properties, and also can withstand with any broad range of temperature and pH (Brown et al., 1999; Khatoon et al., 2014). Both *Nannochloropsis* sp. and *Tetraselmis* sp. are also good source of carotenoid compound (Lubian et al., 2000; Ismaiel et al., 2016). Water quality parameters are considered as important concern in aquaculture to culture fish. Microalgae are mostly found in the marine environments but also found in fresh and brackish water environments. Microalgae is a potential source to stabilize quality of water in an expected outcome (Yang et al., 2021).

In the field of aquaculture, blood parameters are considered as most convenient indicators to determine the health status of farmed and uncultured fish species as these are capable of providing reliable information on possible exposure to mutagens, metabolic stress, deficiencies and chronic stress status before clinical symptoms appear (Bahmani et al., 2001). Haematological profile reflects the physiology and physiological changes of the animals to its external and internal environments as these parameters can vary with season, temperature and environments. Therefore, any change in the external environment can cause a dysfunction of blood and as such have severe effects on the physiological activities such as resistance to disease, metabolism, breeding performance and health condition of the entire body (Esonu et al., 2001).

In this study, Nile tilapia was used as experimental fish as tilapia is one of the most important groups of cultured fish species. They are widely cultivated in the tropical and subtropical countries because of their high commercial value, eat wide range of natural food organisms, can withstand with poor water quality, grow rapidly at warm temperatures and low cost of maintenance. But there is limited information on the nutrition, fatty acid composition and blood parameters of tilapia.

Research was needed to determine if microalgae can replace fish meal in fish feed.

1.1 Objectives of the study:

I. To observe the growth performance and nutritional composition of Nile Tilapia fry, fed with selected microalgae

II. To compare the total carotenoid concentration of Nile Tilapia fry, fed with selected microalgae

III. To evaluate the hemato-biochemical index of Nile Tilapia fry, fed with selected microalgae

Chapter-2: Review of literature

Before conducting any research under a definite experimental procedure, it is important to have a look on the previously conducted research activities on the related topics. Microalgae is an autotrophic microscopic organism considered as a great source of nutrition, carotenoid and immunity etc. A review of literature relevant to the present research work has been given below.

2.1 Microalgae

Microalgae are considered as one of the most important photosynthetic organisms that habituated in different aquatic habitats, which includes ponds, lakes, oceans, rivers, and even wastewater (Khan et al., 2018). These organisms have the ability to tolerate a wide range of salinity, temperatures and pH values with different light intensities; and conditions (Barsanti et al., 2008). Microalgae are two types which are prokaryotic and eukaryotic photosynthetic microorganism. According to the color's microalgae are classified as green, red, blue-green and brown (Graham and Wilcox, 2000). In open water, microalgae contributions are important in producing energy and essential nourishing component for proper development of aquatic organisms (Habib et al., 2003). They also become main live foods for zooplankton such as *Rotifers, Cladocerans* as well as for different fish larvae specially larvae of shrimp (Gallardo et al., 1995).

In aquaculture, microalgae play an important role in aquaculture development. Microalgae are widely used as an ineluctable food source in the field of commercial rearing of all growth stages of mollusks, larval stages of crustaceans and early growth stages of fishes (FAO, 1996). They also can be used as food additive to basic nutrients or as a food coloring. But it must be in proper size for repast, for instance for filter feeders (1-15 micrometer), for grazers (10-100 micrometer) and readily digested (Kawamura et al., 1998).

Microalgae have recently been attracted a considerable level of interest into the whole world. There extensive potential application had been reported in various field including biopharmaceutical, renewable energy, and nutraceutical industries (Barsanti et al., 2008). In today's world microalgal pigments application also recorded widely in industrial field in addition with various research, clinical and pharmaceutical

laboratories (Santiago-Santos et al., 2004). Because of some other properties, it creates a vast feasibility in the field of renewable energy and substitute of biodiesel and natural energy (Parmar et al., 2011). Considering microalgae profile, it is clear that various factors are responsible to influence nutritional profile of microalgae which includes cell size (Fernandez-Reiriz et al., 1989) and ability to digest (Epifanio et al., 1981). For this experiment, two species of tropical marine microalgae have been selected which are *Nannochloropsis* sp., and *Tetraselmis* sp. These marine species have been used deliberately in aquaculture industry especially for growth and larval rearing just because of its nutritional profile (Jeffrey et al., 1994).

2.1.1 *Tetraselmis* sp.

Tetraselmis sp. are unicellular flagellate which is an ovoid body shape and curved in side views. *Tetraselmis* sp. measures 12-14 µm in length and 9-10 µm in width (Mehdi et al., 2015). This species is commonly spherical in shape but elliptical in sometimes with compressed and curved sight. It possesses 4 pair of flagella in both sides. Its eyespot is varying on the basis of the species but present in every cases. Chloroplast is present, two in number in some cases. Two stages are observed one is motile and another is non-motile (Guiry and Guiry, 2017). *Tetraselmis* sp. are mainly used for zooplankton feeding *(Artemia* or *Rotifers)* which are later used for larval feeding of fish larvae (Muller-Feuga et al., 2003). *Tetraselmis* sp. becomes an important source for anti-oxidative substances in pharmacological studies (Laguna et al., 1993) and for their importance in marine eco-toxicological testing (Park et al., 2005). It has also used in plantology (Guiry and Guiry, 2015). Furthermore, Austin et al. (1992) reported the use in antimicrobial field. In modern period is having a great potentiality as probiotics (Irianto and Austin, 2002). This species also considered as good source of vitamin, especially vitamin E and for that referred as animal diet (Carballo- Cárdenas et al., 2003).

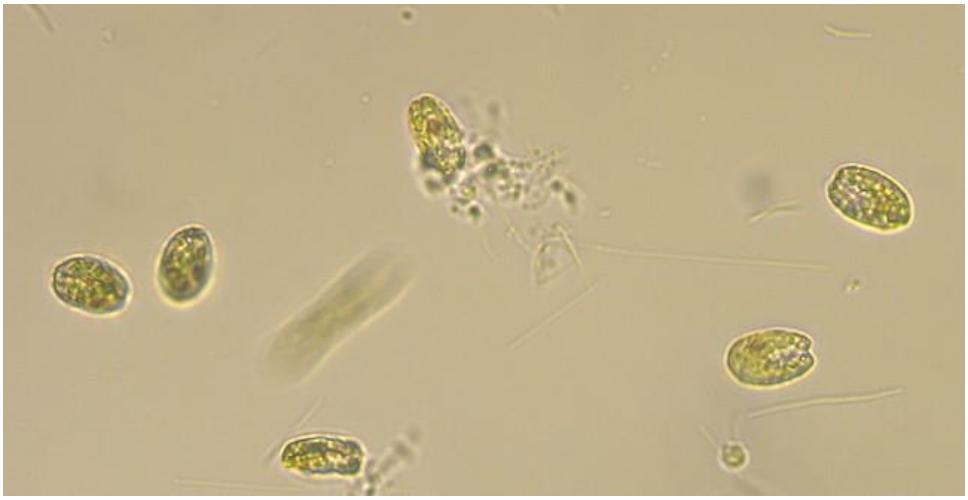


Figure 1: *Tetraselmis sp.* isolated from Cox's Bazar coast.

2.1.2 *Nannochloropsis* sp.

Nannochloropsis sp. are marine, unicellular and free-floating microalga. The cell is sub spherical, with a structure cylindrical diameter. Its chloroplast is moderately developed color tends to yellow to green (Antia and Cheng, 1982). Golgi body and mitochondrion are common in every cell with cytoplasmic lamellate vesicles, a pyrenoid and a cell wall papilla (Hideaki, 2002). According to Ma et al. (2014) the species has plant alike plastids with very simple morphological structure of diameter 3-8 µm. These species are mainly used for zooplankton feeding *(Artemia* or *Rotifers)* which are later use for larval feeding of fish larvae (Malcolm, 1998). *Nannochloropsis* sp. also used in nutritional supplement as it contains high amount of Eicosapentaenoic acid (EPA) (Wan, 2012). *Nannochloropsis* sp. are commonly used in marine hatcheries regulator of water quality (Riquelme and Avendaño-Herrera, 2003). *Nannochloropsis* sp. are commercially cultured for extensive use in the aquaculture industry for growing small zooplankton such as rotifers, copepod, daphnia and *Artemia* (Banerjee et al., 2002) for feeding SPS (Small Polyp Stony) corals and other filter-feeders. In food industry, it is well known as a source of different valuable compounds such as vitamin E (Durmaz, 2007) and pigments; chlorophyll, astaxanthin and canthaxanthin. (Lubian et al., 2000).

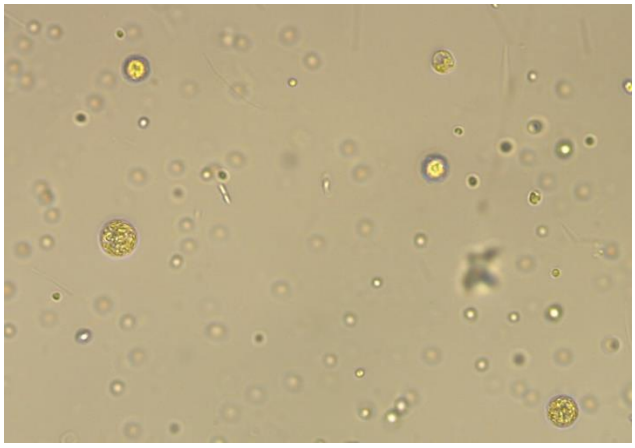


Figure 2: *Nannochloropsis* sp. isolated from Cox's Bazar coast.

2.2 Global Production and Significance of Nile Tilapia

The Nile tilapia is well-suited in wide range of trophic and ecological variations, as well as its adaptive life history characteristics make them a potential culture species worldwide (Trewavas, 1983). Therefore, it has been greatly introduced for aquaculture and sport fishing (Toshio et al., 2002; Walker et al., 2009) and is now found in every country in the tropics. Commercially, after carps, tilapia is the second most important group of wild-captured fish, 851 million tons of wild capture in 2018 (FAO, 2020). Tilapia culture has been popular since 1990s and currently it is one of the world's second most common group of farmed fish species, with a commercial production of 6.031 million tons, corresponding to an estimated value of $11.7 billion (FAO, 2020). In 2020, Nile tilapia (*Oreochromis niloticus*) culture alone was ranked first among the most cultured species in the world, with a total aquaculture production of 6.031 million tons (FAO, 2020). Nile tilapia represents approximately 87.6% of total global tilapia production (FAO, 2020). In 2010, it is anticipated that global Nile tilapia production will reach nearly 15 million tones, with a market value of nearly $20 billion (FAO, 2020). China is by far the largest consumer and producer (about 46% of global production) of tilapia, with a production estimated at 1.62 million tons in 2018, up from 1.45 million tons in 2017 (FAO, 2018). Other main producing countries of farmed tilapia (2017-2018 data) are Indonesia (122,2741 tones), Egypt (105,1444 tones), Bangladesh (344,784 tones) Brazil (317,080 tones), and Philippines (277, 006 tones) (FAO GLOBEFISH, 2018). Projections indicate that Indonesia is most likely to rival China in tilapia production within the next decade (FAO GLOBEFISH, 2018).

Bangladesh has enormous possibility for tilapia farming due to its rapid growth rate and high market value, thus become one of the most widespread culturable species in Bangladesh (Sarker et al., 2018). Production of tilapia, for home or local consumption and for export, has risen enormously in the last few decades. A significant number of farmers in the rural area have been involved in tilapia farming due to its high productivity. Therefore, for viable and sustainable aquaculture, it is urgent need to identify the important and less expensive components, growth and survivability influencing ingredients can be used in culture system.

2.3 Biology and Ecology of Nile Tilapia

A species of tilapia, a cichlid fish, is commercially known as Nile tilapia, mango fish, niloticus, or boulti. The word, "Tilapia" is a derivative of an African Bushman word simply meaning fish. Fishes belonging to the family Cichlidae is referred to as Tilapias which is an extremely hardy fish tolerating wide range of water parameter such as hardness, pH, temperature, and also dissolved oxygen. This species has their tolerance to hypoxia and will survive in low oxygen levels with high biochemical oxygen demand (BOD). Also, Tilapia is a hardy, prolific, fast growing tropical fish, and it can survive on a diversity of food (Richmond, 2015).

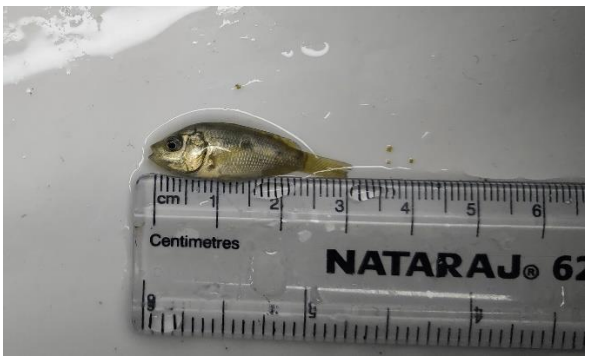


Figure 3: Nile tilapia fry collected from Niribili Tilapia Hatchery, Coxes Bazar.

2.3.1 Biological Features

Generally, Nile tilapias are brownish or grayish in color, sometimes with indistinct banding on their body, and the tail is vertically striped. During breeding, males become reddish, especially on their fins (Nico et al., 2019). Sometimes they are confused with the blue tilapia (*O. aureus*), which doesn't have the striped tail pattern and in dorsal fin they have a red edge that is gray or black in Nile tilapia. They can also be separated by

meristic. The length of Nile tilapia can reach up to 60 cm (Froese et al., 2015) and weight can exceed 5 kg (11 lb). As average of tilapia, males reach a larger size and grow faster than females. Nile tilapia can live for long time, almost more than 10 years (Nico et al., 2019).

2.3.2 Habitat and Distribution

The freshwater cichlid, Nile tilapia is native to the Nile River basin and the south-western Middle East (Trewavas, 1983). Mostly for farming purposes, they have been introduced in all continents (more than 50 countries) except Antarctica (Sheikhzadeh et al., 2012). It was introduced in Bangladesh from Thailand in 1954 (Santhosh and Singh, 2007; Shields et al., 2012). Mostly the natural habitat of Nile tilapia are different freshwater bodies like ponds, lakes, canals, streams, rivers and ranging from sea level to an altitude of 1,830 m (Froese et al., 2015). As they are euryhaline species, so can also be found in brackish water, but is unable to survive long-term in full salt water (Froese et al., 2015). They can tolerate water temperatures between 8 and 42 °C (46 and 108 °F), although typically above 13.5 °C (56.5 °F) with some variations depending on the population. Although they can survive in a wide range of temperature, generally breeding only occurs in 24 °C (Nico et.al., 2019).

2.3.3 Reproductive Biology

Nile tilapias are maternal mouth-brooders. Typically, the male build nest on breeding purpose where mass spawning of a brood occurs. Then the male fertilized the eggs and after that the fertilized eggs are picked up by the female in their mouth even after hatching. Female tilapias can produce several hundred to several thousand young per spawn. They breed within months after birth. Moreover, the younger Nile tilapia leads to high birth and turnover rates.

In the presence of other females, the female tilapia exhibit shortened inter-spawning intervals. Those female tilapias that abandon their young to the care of a male gain this advantage of increased inter-spawning periods. The reason of this reproductive mechanism is to increase the advantage of females allowing them more opportunities to spawn as they don't have to take care of the young ones anymore (Tacon, 1996). In case of males, the more dominant males get the reproductive advantages as they have higher levels of the gonadotropic hormone. Thus, for larger sperm production more

successful males are selected. Also, dominant males have both the best territory in terms of resources and the greatest access to mates (Pfennig et al., 2011). Visual communication between mates both stimulates and modulates reproductive behavior between partners such as courtship, spawning frequency, and nest building (Castro et al., 2009).

2.3.4 Feeding Habit

The Nile tilapia is mostly herbivore, but with omnivorous tendencies, especially when young (Froese et al., 2015). Depending on their feeding behavior, they mostly feed on phytoplankton and algae, and in some populations other macrophytes also an important feed source (Diallo et. al., 2020). Some other recorded food items are detritus and aquatic insect larvae (Froese et al., 2015), including those of mosquitoes. However, outside of their native range, it often becomes invasive, threatening more localized species (Froese et al., 2015). Similar to trout and salmon, Nile tilapia typically feeds during day time showing that light is a main factor in their feeding activity. As they have high reproduction rate, overpopulated culture will require night feeding to provide back up in competition among individuals for necessary nutrients. Based on a recent study it was observed that size dimorphism between the sexes is depended on food conversion efficiency rather than the amounts of food consumed. That's why, the male grows larger than the female consuming same amount of food because of their higher efficiency of converting food to body weight (Toguyeni et al., 1997).

2.3.5 Nutritional Profile of Nile Tilapia

Tilapia has become a popular food item, mainly because of its high nutritional value, mild taste, and low expense relative to other finfishes. In America, tilapia has been appeared in top 10 seafood list (2002) and 5th in 2008 (NFI, 2010). Tilapia contains high protein, phosphorus, potassium, vitamin B-12, and is low in fat and saturated fat, omega-3 fatty acids, calories, carbohydrates, and sodium.

2.4 Nutritional Composition of Microalgae

2.4.1 Proximate Composition

Microalgae species have shown different proximate nutritional profile because of different culture pattern (Brown et al., 1997). The nutritional composition of microalgae depends on their environmental conditions, growth rates or the life cycle (Richmond, 1986). It is well known about the effect of intensity of light, fluctuation of temperature, salinity range and media types on the growth and proximate composition of microalgae (Brown et al., 1997). Overall, microalgae grown in mature harvest condition typically contain protein ranges 30-40%, lipid ranges 10-20% and carbohydrate ranges 5-20% (Renaud et al., 1999). Brown et al. (1997) gives a wider range for the level of protein, lipid and carbohydrate which are 6-52%, 7- 23% and 5-23% respectively.

Gbadamosi and Lupatsch (2018) concluded that *Nannochloropsis salina* can replace fish meal and soyabean meal up to 100 % in tilapia feeds without showing any negative results in the growth, survival and health of the fish. Tulli et al. (2012) showed that dried *Tetraselmis suecica* is able to replace up to 20% of fish meal without hampering the growth performance of sea bass and has the potential to become an alternative dietary ingredient to be used in organic feed production.In general, mixed-microalgal diets provide more balanced nutrition (e.g., lipids, proteins, carbohydrates and essential fatty acids) than single-microalgal diets and thus are more likely to meet the nutritional requirements of larval/juvenile bivalves produced in hatcheries (Fujii et al., 2010). With the increasing of microalgae in diet, the nutrition level in the fish body is increasing as their digestibility. Various studies suggest that herbivores require comparatively less amounts of proteins compared to carnivores (Toguyeni et al.,1997). Sorensen et al. (2017) concluded that *Nannochloropsis oceania* is able to influence high growth and protein content in Atlantic salmon in replacement of 10% of fish meal. Sarker et al. (2016) found high protein efficiency ratio in Nile tilapia fish due to 100% replacement of fish oil with marine microalgae *Scizochytrium* sp.

2.4.2 Fatty Acid

Nannochloropsis sp. regarded as a potential source of essential ω-3 PUFA, EPA (20:5ω-3) (Sukenik et al., 1990; Renaud et al., 1991; Renaud et al., 1994). Gbadamosi and Lupatsch (2018) concluded that fatty acid profile of *Nannochloropsis salina*

increases the ω-3 PUFA in edible fish and enhances the essential nutrient components for diet of human beings. *Tetraselmis* sp. are unicellular flagellated chlorophytes with rapid growth rate and can withstand with broad range of temperature and pH (Khatoon et al., 2014). They are known to have a promising nutritional profile containing sufficient amount of protein, carbohydrate, lipid and fatty acids which are vital for cultured organisms. According to Pereira et al. (2019) *Tetraselmis* sp. contain PUFA, EPA and α-linolenic acids, which are important for different nutritional applications. *Tetraselmis chuii* is a widely used microalgae that is thought to be an excellent source of long-chain PUFAs, particularly EPA (Meseck et al., 2005; Zaki and Saad, 2010).

2.5 Carotenoid Content

Carotenoids are considered as one of the most important group of pigments which provides various colors like yellow, red and orange to fish, crustaceans, animals and plants skin and tissues (Kop and Durmaz, 2008). Different plant species, various fungi, heterotrophic bacteria and photosynthetic prokaryotes can produce lipophilic pigments regarded as carotenoids. They highly contribute in photosynthetic activity because carotenoid contains different pigments which harvests lights and prevents photo oxidation (Hirschberg, 2001; DellaPenna and Pogson, 2006; Walter and struck, 2011). Many fish deposit carotenoids in their integuments and gonads but in case of salmonids, they accumulate carotenoids in muscle. According to Torrissen and Nsevdal (1988) reported that, in matured fish species high quantity of carotenoids are observed in its integument and ovaries, almost 90% carotenoid was also observed as free form in fish flesh. Different aquatic species like fish and other animal species could not able to produce carotenoid pigments in their skin and muscle tissues as a result they are completely dependent on dietary carotenoids which regulates coloration in fish and other animal species. Teimouri et al. (2013) concluded that dietary supplements with *Spirulina platensis* increased pigmentation in rainbow trout.

2.5.1 Application of Carotenoid

As nutritional supplement there is an increasing demand for natural carotenoids where most carotenoids are chemically synthesized (Jin et al., 2003). This is happened because of dominating β-carotene in synthetic carotenoids and more cis forms in natural forms (Ton Laar et al., 1996). β-carotene has 10-12% less absorption rate than cis form. It is recently used in different food industry as colorant and food additives because of its

provitamin activity. In addition, β-carotene has antioxidant and anticancer properties (Becker, 2004). In the field of market applications β-carotene used as provitamin A (retinol) in food and animal feed, food coloring agent, as an additive (Johnson and Schroeder, 1995; Edge et al., 1997). In the field of nutraceuticals, cosmetics, food and feed industries xanthophyll, astaxanthin has many applications. Presently carotenoid has a major application as pigmentation source in aquaculture (Guerin et al., 2003; Cysewski and Lorenz, 2004). Recently Gurein et al. (2003) and Higuera-Ciapara et al. (2006) have been claimed carotenoid as potential element regarding health and nutritional constituents.

2.6 Microalgae in Fish Hemato-biochemical Index

Counting the cells of blood provides a certain index which is maintained by fish in a certain range. Hassaan et al. (2020) depicted that dietary supplementation with extracted bioactive compounds from *Spirulina* β-carotene or phycocyanin showed a significant result in the enhancement of the total serum protein, albumin, and globulin. The malic acid and *B. subtilis* mixture can improve both growth and health through its positive impact on the GI (gastrointestinal) tract, liver function, blood parameters and non-specific immune responses (Hassaan et al., 2017).

Chapter-3: Materials and Methods

This chapter deals with the methods that are followed and materials that are used to observe the effects of different dietary levels of microalgae. This study was conducted in two phases. In the first phase, two selected marine microalgae species were cultured. Dried biomass of selected microalgae was kept in refrigerator for further use during feed preparation. In the second phase, growth, water quality, proximate composition, fatty acid composition, total carotenoid concentration, hemato-biochemical index of fry Nile tilapia were determined.

3.1 Experimental Site and Collection of Microalgae

The feeding trial experiment on tilapia was done at the Wet laboratory of Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University with maintaining proper precautions. The duration of tilapia fry feeding trial was 8 weeks. Fry of tilapia was collected from a commercial hatchery named "Niribili Tilapia Hatchery", situated at Cox's Bazar. Two different strains of marine microalgae, *Tetraselmis* sp. and *Nannochloropsis* sp. were used in this experiment. They were collected from the live feed research corner of Department of Aquaculture, Chattogram Veterinary and Animal Sciences University.

3.2 Preparation of Conway Medium

Pure Conway medium was used for the culture of *Tetraselmis* sp. and *Nannochloropsis* sp. Conway medium constitutes with different macronutrients, metal solutions and vitamins which are shown in Table 1 (modified from Tompkins et al.,1995). One mL of solution A, 0.5 mL of solution B and 0.1 mL of solution C were added with autoclaved and sterilized seawater to make 1 L Conway media.

Table 1 Utilized Conway medium for microalgae cultivation (modified from Tompkins et al.,1995).

Macronutrients-Solution A	
Molecular formula and chemical name	**Quantities**
$NaNO_3/KNO_3$ (Sodium/Potassium nitrate)	100.00 g/116.00 g
H_3BO_3 (Boric acid)	33.60 g
$C_{10}H_{16}N_2O_8$ (EDTA disodium salt)	45.00 g
$NaH_2PO_4.4H_2O$ (Sodium di-hydrogen orthophosphate)	20.00 g

$FeCL_3.6H_2O$ (Ferric chloride hexahydrate)	1.30 g
$MnCL_2.4H_2O$ (Manganese (II) chloride tetrahydrate)	0.36 g
Distilled/ deionized water	1 L
Trace metal solution-Solution B	
Molecular formula and chemical name	**Quantities**
$ZnCl_2$ (Zinc chloride)	2.10 g
$CuSO_4.5H_2O$ (Copper (II) sulfate pentahydrate)	2.00 g
$CoCl_3.6H_2O$ (Cobalt (II) chloride hexahydrate)	2.00 g
$(NH_4)_6MO_7O_{24}.4H_2O$ (Ammonium molybdate tetrahydrate)	0.90 g
Distilled/ deionized water	1 L
Vitamin solution-Solution C	
Molecular formula and chemical name	**Quantities**
Vitamin B1, Thiamine	0.20 g
Vitamin B12, Cyanocobalamin	0.01 g
Distilled/ deionized water	0.1 L

3.3 Mass Culture of Microalgae

Tetraselmis sp. and *Nannochloropsis* sp. microalgae mass culture was performed in 20 L transparent plastic jar in large scale. Conway medium was used for mass culture so that culture media can be prepared (Table 1). From an initial starter culture volume of 20 mL to 20 L the culture volume of microalgae was scaled up progressively. For batch culture, at first 20 mL of microalgal stock was combined with 30 mL medium in individual flask and total volume of culture was 50 mL. After that, the culture volume was gradually expanded like, 250 mL, 500 mL, 1 L and at last scaled up into 20 L big plastic tank, with batch culture. The cultures were shifted into next batch during the exponential period of development (Islam et al., 2021). After reaching to stationary phase (Islam et al., 2021), microalgae cells were centrifuged at 5000 rpm for 5 minutes by using centrifugation machine (HERMLE Z 206A, Germany). After centrifugation the wet biomass was collected. Then the wet biomass was dried at 40°C temperature for overnight by using a hot air oven (Natural Convention Oven LNO-150, JSR Korea) and a mortar and pestle was used to grind oven-dried microalgae biomass into fine

particles (0.4-0.5 mm in diameter). After that, the collected powdered microalgae were stored until further use for feed preparation in normal freezer at 4°C. Mass cultivation was continued until sufficient dried biomass of both algae were attained to conduct the feeding trial.

3.4 Experimental diets

Five diets were prepared using different levels of algal biomass (0%, T25%, T50%, N25%, N50%). During diet formulation 25% and 50% fish meal were replaced by the dried biomass of *Nannochloropsis* sp. and *Tetraselmis* sp. and there was no replacement in control diets. All feeds were formulated with commonly used ingredients (Amira et.al., 2021). Proximate composition of experimental diets and microalgae are shown in Table 2.

Table 2 Diet formulation and percentages of proximate composition for *Oreochromis niloticus* diet (% dry weight basis) (Amira et.al., 2021).

	Diet formulation				
Constituents	**CF**	**T25**	**T50**	**N25**	**N50**
Commercialized fish meal	67.55	50.67	33.77	50.67	33.77
Nannochloropsis sp.	_	_	_	16.88	33.78
Tetraselmis sp.	_	16.88	33.78	_	_
Corn flour	9.65	9.65	9.65	9.65	9.65
Wheat flour	9.65	9.65	9.65	9.65	9.65
Rice bran	9.65	9.65	9.65	9.65	9.65
Vitamin mixture	1	1	1	1	1
Mineral mixture	1	1	1	1	1
Dicalcium phosphate	1	1	1	1	1
Molasses	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100
Proximate composition (%) of experimental diet					
Carbohydrate	20.43	23.19	26.22	22.33	23.18
Protein	40.00	38.00	36.90	36.03	33.45
Lipid	11.21	12.29	13.11	14.37	16.22
Proximate composition (%) of microalgae					

	Nannochloropsis **sp.**	*Tetraselmis* **sp.**
Carbohydrate	17	22
Protein	49	57
Lipid	25	19

Abbreviations: CF-control feed with no replacement of fish meal with microalgae; T25-treatment with 25% replacement of fish meal with *Tetraselmis* sp.; T50-treatment with 50% replacement of fish meal with *Tetraselmis* sp.; N25-treatment with 25% replacement of fish meal with *Nannochloropsis* sp.; N50-treatment with 50% replacement of fish meal with *Nannochloropsis* sp.

3.5 Analysis of Proximate Composition

Chemical analysis methods were followed to determine the proximate composition of microalgae and experimental diet.

3.5.1 Determination of Protein Content

Method provided by Lowry et al. (1951) was followed to determine the protein content. Oven-dried 5 mg powdered sample was taken into a test tube besides for solution preparation, 25 ml of deionized water were added and mixed. Then for protein analysis, 0.5 ml aliquot of individual sample was extracted from the prepared 25 ml sample solution. Two reactive solutions for mixed reagent preparation (reactive 1: potassium sodium tartarate tartrate 1% and reactive 2: sodium carbonate 2 g for each 100 ml 0.1 N NaOH) were made previously. By mixing 1 ml of reactive 1 and 50 ml of reactive 2, mixed reagent was prepared. Then with 0.5 ml of 1 N sodium hydroxide, 0.5 ml of sample was added and for 5 minutes it was kept in 100°C in water bath. From the prepared mixed reagent, 2.5 ml was taken and added to the tube after cooling it for 10 minutes. The mixed solution was added with 0.5 ml Folin reagent and kept in a dark condition for 30 minutes. The reading of absorbance for the prepared solution was obtained by using spectrophotometer (T80 UV/VIS Spectrophotometer, UK) at 750 nm wavelength.

3.5.2. Determination of Lipid Content

Method provided by Bligh and Dyer (1959) was followed to determine the lipid content. Aluminum plates for each sample was briefly prepared and labelled. For each labelled aluminum plates initial weight was noted. Sample was diluted 5 times using distill water, after previously weighted 50 mg of sample was taken into a centrifuge tube. A tissue homogenizer was used to homogenize added sample containing 3 ml 1:2 chloroform: methanol (v/v). All the centrifuge tubes were centrifuged for 4 minutes at

1000 rpm. By using a Pasteur pipette the supernatants were extracted into clean centrifuge tube then reserved it into ice. In the similar way, 3 ml aliquot of methanol: chloroform (v/v) at the ratio of 2:1 was consistently mixed with the solution. The supernatants were moved to the previous supernatant tubes after the tubes were again centrifuged. Supernatant which was combined with solutions then added with 0.9 % of 1.5 ml NaCl and mixed gently using a vortex mixture (VM-10). At 4 °C all the tubes were then stored for an hour in the freezer. For 10 minutes at 4 °C temperature, the tubes were then centrifuged after an hour. From the upper layer, chloroform and methanol were removed, meanwhile plates of aluminum had been used for the low layer transportation. For evaporation of the solvent at 40 °C, a hot air oven was used. Then the weight of aluminum plate was noted so that the final weight of plate can be obtained. Lastly, from the final weight of plate the initial weight was deducted in order to record the samples final lipid weight.

3.5.3. Determination of Carbohydrate Content

To determine carbohydrate content the method of Dubois et al. (1956) was followed. Five mg of oven-dried powdered microalgae sample was taken for individual analysis while adding deionized water 25 ml solution was prepared. Concentrated sulphuric acid (H_2SO_4) and phenol solution (5%) were also prepared. In the glass test tube from the prepared sample, aliquot of 1 mL was extracted. At that point, for cooling the added aliquot in test tube containing 1 mL 5 % phenolic solution and 5 ml of sulphuric acid (conc.) were kept into the ice bath. The reading of absorbance for the prepared solution was obtained by using spectrophotometer (T80 UV/VIS Spectrophotometer, UK) at 488 nm wavelength.

3.6 Collection of Fish and Experimental Design

Fourteen days old 270 Nile tilapia fish fry (mean individual weight 0.023 ± 0.0001 g) were used to conduct this feeding trial experiment. All the fries were distributed randomly into fifteen four-sided clear glass aquaria (45×30×30 cm) and encompassing water holding capacity up to 30 L. The stocking density was 18 fish/tank within 18 L water. Fish were acclimatized in a large tank at laboratory condition for 2 days prior to the stocking. Continuous aeration was supplied to maintain the sufficient oxygen level in tank and commercial starter feed was given as feed to the fish at the time of conditioning. After conditioning period, 18 fish in each tank were stocked according to the experimental design. Five treatments were designed namely CF, T25, T50, N25, N50 in triplicate form to conduct this experiment and tilapia fry were fed with diet

containing *Tetraselmis* sp. and *Nannochloropsis* sp. along with a control feed with no microalgae. All the five categories of feed were grinded and provided 4 times in a day (at 8 AM, 11 AM, 2 PM and 5 PM) according to the 15% of the total body weight of the fish. Excreta and leftover feeds were removed from the bottom of each aquarium through siphoning on a regular basis. One third volume of culture water was exchanged daily from each experimental tank. Aeration was maintained continuously by using aerator throughout the culture system.

3.7 Analysis of Water Quality Parameters

To analyze variations in physiochemical parameters of water quality during the feeding experiment, daily analysis of DO (dissolve oxygen), pH, temperature and weekly analysis of Total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N), and soluble reactive phosphorous (SRP) were examined. Digital glass thermometer (SARAAN SCIENTIFIC INDUSTRIES), pH meter (pHep-HI98107, HANNA, India), and DO meter (Lutron A20 DO5509 Dissolve Oxygen Meter, Taiwan, China) were used to measure the readings of water temperature, pH, and DO in the culture tanks. Total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N), and soluble reactive phosphorous (SRP) were determined using the chemical method of Parsons et al. (1984). Thirty ml of mixed water sample from each tank was taken and filtered with a Whatman GMF Circles 4.7 cm for analysis.

3.7.1 Total Ammonia Nitrogen (TAN)

Ten ml of water sample was taken into the test tube and then 0.4 ml of phenol solution (C_6H_6O) (20 g of analytical grade phenol dissolved in 200 ml of 95 % (v/v) ethyl alcohol) and 0.4 ml of sodium nitroprusside ($C_5FeN_6Na_2O$) (1 g sodium nitroprusside dissolved in 200 ml of MiliQH_2O) were added into test tube. 1 mL of oxidizing solution was subsequently added. A mixture of 100 ml alkaline reagent (100 g sodium citrate and 5 g sodium hydroxide in 500 ml MiliQH_2O) and 25 ml sodium hypochlorite (NaClO) solution was used for the preparation of an oxidizing solution. Then the test tubes were covered with parafilm and kept at room temperature (24-27 ºC) for 1 hour. The prepared samples were then measured at 640 nm wavelength with spectrophotometer.

3.7.2 Nitrite nitrogen (NO_2-N)

Ten ml of water sample was taken into test tube and then 0.2 ml of sulfanilamide solution ($C_6H_8N_2O_2S$) was added. The sulfanilamide solution was prepared by dissolving 5 g of sulfanilamide in a mixture of 50 ml of concentrated hydrochloric acid

(HCL) and diluted to 500 ml with MiliQH_2O. After 8-minute, addition of 1 ml of NED reagent ($C_{12}H_{14}N_2$) (0.5 g of the N-(1-napthyl)-ethylenediamine dihydrochloride dissolved in 500 ml of MiliQH_2O) to the tube was done and mixed instantly with vortex mixture (VM-10). Within (10 minutes to 2-hours) afterwards extinction prepared samples were measured at 543 nm wavelength with spectrophotometer.

3.7.3 Soluble Reactive Phosphorus (PO_4-P)

Test tube containing 10 ml of water sample was supplemented with 1 ml of mixed reagent. By mixing 100 ml of 0.02 M ammonium molybdate ($(NH_4)_6MO_7O_{24}.4H_2O$), 250 ml sulfuric acid (H_2SO_4), 100 ml of 0.31 M ascorbic acid ($C_6H_8O_6$), and 100 ml of 0.002 M potassium antimonyl-tartrate ($C_8H_{10}K_2O_{15}Sb_2$) mixed reagent was prepared. After 5 minutes and preferably within the first 2-3 hours, extinction was measured at 885 nm wavelength by using spectrophotometer.

3.8 Fish Sample Preparation

At first, whole fish samples were oven dried (Natural Convention Oven LNO-150, JSR Korea) at 40 ^{0}C for overnight. Oven dried fish samples were grounded using a mortar and pestle to form into powdered format for the analyses of protein, carbohydrate, lipid content and fatty acids of Nile tilapia fish.

3.8.1 Determination of Proximate Composition of Nile Tilapia

To determine carbohydrate content of whole Nile Tilapia fish tissue the method of Dubois et al. (1956) was followed. Method provided by Lowry et al. (1951) was followed to determine the protein content. The reading of absorbance for the prepared sample solution for protein and carbohydrate was obtained by using spectrophotometer (T80 UV/VIS Spectrophotometer, UK) at 750 nm and 488 nm wavelength respectively. Method provided by Bligh and Dyer (1959) was followed to determine the lipid content.

3.8.2 Analysis of Fatty Acid Composition of Nile Tilapia

The direct methylation techniques of Divakaran and Ostrowski (1989) was followed to prepare fatty acid methyl esters (FAMEs). The fatty acid analysis was done in "Nutrition and Processing Laboratory" at department of "Fishing and Post-Harvest Technology", Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. Firstly, total lipid content of sample was extracted out by using Soxhlet apparatus. Diethyl ether has been utilized as a lipid extraction solvent and 60°C temperature was maintained at the final stage of lipid extraction. This extracted lipid

was the final lipid sample and used for the fatty acid methyl esters analysis. In each sample, 1.5 mL methanolic NaOH was added and kept into the sonicator (Ultrasonic bath 621.06.010) at 80 ^{0}C for 5 minutes. After sonication samples were kept out from the sonicator bath and 2 mL boron-trifluride methanol was added in each sample. Again, samples were kept into the sonicator at 80 ^{0}C for 30 minutes. After that, iso-octan 1 mL was added into each sample vial and shook gently for a while. Then 5 mL saturated NaCl was also added and after shaking waited for a while. After a while 2 clear layer was separated. The upper layer was collected by using micropipette and kept in 1 mL GCMS (Gas Chromatography and Mass Spectrophotometry) vial so that vial can be used for fatty acid analysis in GCMS. A gas liquid chromatograph (GC-2010 Plus, Shimadzu, Japan) having high sensitivity FID with clean detector gas flows and a capillary column (Film thickness 0.25 μm, internal diameter 0.25 mm, length 25 m) was used for the analysis of the FAMEs of the samples. The injection volume was 1 μL. The column temperature was: firstly (50-280) ^{0}C at 3 ^{0}C /min and then (45-180) ^{0}C at 2 ^{0}C/min. Compared to retention periods of established standards, individual FAME peaks were identified. At first, each fatty acid quantities were expressed as ppm and later converted into percentages of total fatty acids.

3.9 Determination of Total Carotenoid Concentration of Nile Tilapia

Fish were sampled after 8 weeks of feeding trial and 3 fish/tank were taken in order to determine the pigment density, and the level of color enhancement of fish tissue due to microalgae fed diet. Fish were starved for 24 h before sampling. Olson (1979) method of pigment extraction and determination was followed. One gram of Nile tilapia fish body tissue (except head and alimentary canal) was taken as a sample from each tank and kept in a 10 mL screw capped clear glass vials. And then, 2.5 g of anhydrous sodium sulphate (Na_2SO_4) was added into the glass vial. Then the sample was homogenized with hand-homogenizer. 5 mL chloroform was added in that vial and left at 0 ^{0}C for overnight. When a clear 1-2 cm layer was formed above the caked residue, optical density was taken at 380 nm, 450 nm, 470 nm and 500 nm by spectrophotometer (T80 UV/VIS Spectrophotometer, UK) so that the maximum reading range for calculation can be compared. Before being read, 0.3 ml chloroform aliquots from each glass vial were collected and diluted to 3 mL 100% ethanol. In a similar way for comparison a blank was prepared. For the computation, the wavelength with the highest absorption

was utilized. The maximum wave length absorbance was calculated by following formula:

Total carotenoid concentration (µg/g wet weight) = {Absorption at maximum wave length (nm) / (0.25 sample weight (g)} ×10

Where,

Dilution factor =10;

Extinction coefficient= 0.25

3.10 Analysis of Blood Parameters of Nile Tilapia

From each culture tank five fish were randomly selected towards the end of the feeding trial and to determine the blood parameters, clove oil (eugenol solution) has been used to anaesthetize fish. One mL sterile syringe has been used to collect the blood samples from the caudal vein of the fish. For hematology and serum biochemical parameter analysis, collected blood samples were quickly shifted into non-heparinized 2 ml (EDTA K3 PROVEN vacuum tube) tubes besides also in heparinized (CURE clot activator tube) tubes correspondingly. Collected non-heparinized blood samples were stored in refrigerator at 4 ^{0}C, until analysis and heparinized blood samples were centrifuged at 2000 rpm for 10 minutes at 4 ^{0}C (Reyes-Becerril et. al., 2014). After that, collected blood plasma was shifted into Eppendorf tube (1.5 ml) via micropipette and stored at -20 ^{0}C in refrigerator until further analysis. Using Hematology analyzer (NIHON KOHDEN, India) hematological analysis of blood parameters such as, Red blood cells (RBC), Hemoglobin (Hb), Hematocrit (Hct), White Blood Cells (WBC), Lymphocytes (LYM) and Platelets (PLT) were accomplished. In the meantime, biochemistry analyzer (Humalyzer 3000, Germany) was used to determine biochemical parameters of fish blood serum like: total serum protein, albumin, globulin, triglyceride, cholesterol, blood glucose, urea and blood urea nitrogen (BUN).

3.11 Determination of Growth Parameters of Nile Tilapia

Growth parameters like, weight gain, condition factor, specific growth rate, feed conversion ratio and survival of each treatment of the Nile tilapia was determined (Amira et.al., 2021) by the following formulas:

A. Weight gain (g) = final weight – initial weight (Schmalhousen, 1926)
B. Condition factor = (weight (g)/Length (cm)^3) × 100 (Pauly, 1983)
C. Specific growth rate (% / day) = (Ln (W_t)-Ln (W_i))/t × 100 (Ricker, 1990)

Where, t is the time in days. Ln(w_i) is the natural logarithm of the initial weight and Ln(w_t) is the natural logarithm of the final weight at time t.

D. Feed conversion ratio = amount of dry food intake (g)/fresh weight gain in fish (g) (Utne, 1978)

E. Survival rate (%) = (Number of fishes at the end of the experiment / Number of fishes at the beginning of the experiment) × 100

3.12 Data Analysis

All the obtained data is presented as mean ± SE (standard error). IBM SPSS software (v.26) tool was used to analyze the data of growth performance, water quality, pigmentation, hemato-biochemical index, proximate and fatty acid composition. By Kolmogorov-Smirnov test, normality of the data was confirmed. The variation in homogeneity was checked through the Levene test. Finally, ANOVA (one way analysis of variance) test was conducted to confirm the significancy ($p < 0.05$) of data obtained through the experiment and means of different treatments were compared through multiple range test of Duncan.

Chapter-4: Results

4.1 Growth Performance of Nile Tilapia

Growth parameter data of initial body weight (IBW), final body weight (FBW), weight gain (WG), initial length (IL), final length (FL), initial and final condition factor (K), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate of Nile tilapia are depicted in Table 3. Nile tilapia fish fry was fed on diet formulated with microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. for growth parameter study against a control treatment to evaluate the growth performance variation.

During the study fish accepted formulated five categories of diet and growth performance was varied significantly ($p < 0.05$) among the five-treatment group (Table 3). The inclusion of *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae in diet did not increase the WG, K, FCR and SGR of Nile tilapia fry in comparison to reference group CF. Notable differences in survival percentage were also noticed and the survival of each treatment with microalgae inoculated diet was significantly higher ($p < 0.05$) than control treatment (Table 3). Among all the treatment group T25 showed the highest survivality (92.59 ± 1.85%) and the lowest survival was recorded in control group (61.11 ± 3.20%). However, N25, T50 did not vary significantly ($p < 0.05$) and showed similar results as T25.

Table 3 The effect of different concentrations of *Nannochloropsis* sp. and *Tetraselmis* sp. fed diet on growth performance of Nile tilapia fry.

Parameters	Treatment				
	CF	**N25**	**N50**	**T25**	**T50**
IBW (g)	0.023 ± 0.00^{a}	0.023 ± 0.00^{a}	0.0233 ± 0.00^{a}	0.022 ± 0.00^{a}	0.022 ± 0.00^{a}
FBW (g)	2.12 ± 0.00^{a}	1.33 ± 0.00^{d}	1.004 ± 0.00^{e}	1.87 ± 0.00^{b}	1.53 ± 0.00^{c}
WG (g)	2.10 ± 0.00^{a}	1.31 ± 0.00^{d}	0.98 ± 0.00^{e}	1.85 ± 0.00^{b}	1.50 ± 0.00^{c}
IL (cm)	0.9 ± 0.01^{a}	0.9 ± 0.02^{a}	0.9 ± 0.03^{a}	0.9 ± 0.03^{a}	0.9 ± 0.03^{a}
FL (cm)	4.88 ± 0.01^{a}	4.31 ± 0.01^{d}	3.95 ± 0.03^{e}	4.78 ± 0.02^{b}	4.51 ± 0.01^{c}
Initial K (g/cm^3)	3.13 ± 0.10^{a}	3.21 ± 0.31^{a}	3.23 ± 0.29^{a}	3.11 ± 0.27^{a}	3.14 ± 0.28^{a}
Final K (g/cm^3)	1.82 ± 0.01^{a}	1.66 ± 0.01^{bc}	1.63 ± 0.03^{c}	1.71 ± 0.02^{b}	1.67 ± 0.01^{ab}
SGR (%/day)	8.05 ± 0.01^{a}	7.24 ± 0.01^{d}	6.72 ± 0.01^{e}	7.87 ± 0.03^{b}	7.52 ± 0.03^{c}

FCR	2.4 ± 0.01^{e}	3.4 ± 0.00^{b}	4.3 ± 0.01^{a}	2.7 ± 0.01^{d}	3.1 ± 0.01^{c}
Survival (%)	61.11 ± 3.20^{c}	87.03 ± 1.85^{ab}	85.18 ± 1.85^{b}	92.59 ± 1.85^{a}	90.74 ± 1.85^{ab}

Mean ± SE (standard error) along with different small uppercase letters of growth parameters within each column are statistically significant ($p < 0.05$) and determined by the multiple range test of Duncan.

4.2 Water Quality

No significant differences were observed in the results of DO, pH, temperature of different treatments. Means (± SE) of DO, pH, temperature, TAN, NO_2-N and SRP of different treatment is shown in Table 4. Values of TAN, NO_2-N, SRP of CF, N25, N50, T25 and T50 treatments have significant differences among each other ($p < 0.05$) and in comparison, to control treatment. Highest TAN, NO_2-N and SRP concentration obtained by CF (0.66 ± 0.01 mg/L), (0.51 ± 0.01 mg/L) and (0.15 ± 0.00 mg/L) respectively (Amira et.al., 2021). In contrast, lowest TAN concentration was recorded in T25 (0.46 ± 0.00 mg/L) treatment. Similar results were obtained in N25 (0.54 ± 0.00 mg/L) and T50 (0.54 ± 0.00 mg/L) in case of TAN concentration. Lowest NO_2-N concentration reported in T25 (0.42 ± 0.01 mg/L), T50 (0.42 ± 0.01 mg/L) and SRP concentration was reported in N25 (0.10 ± 0.00 mg/L), T25 (0.10 ± 0.00 mg/L), treatment respectively (Table 4) which showed significantly no difference ($p < 0.05$).

Table 4 Results of physical and chemical water quality parameters tested during feeding experiment (Amira et.al., 2021).

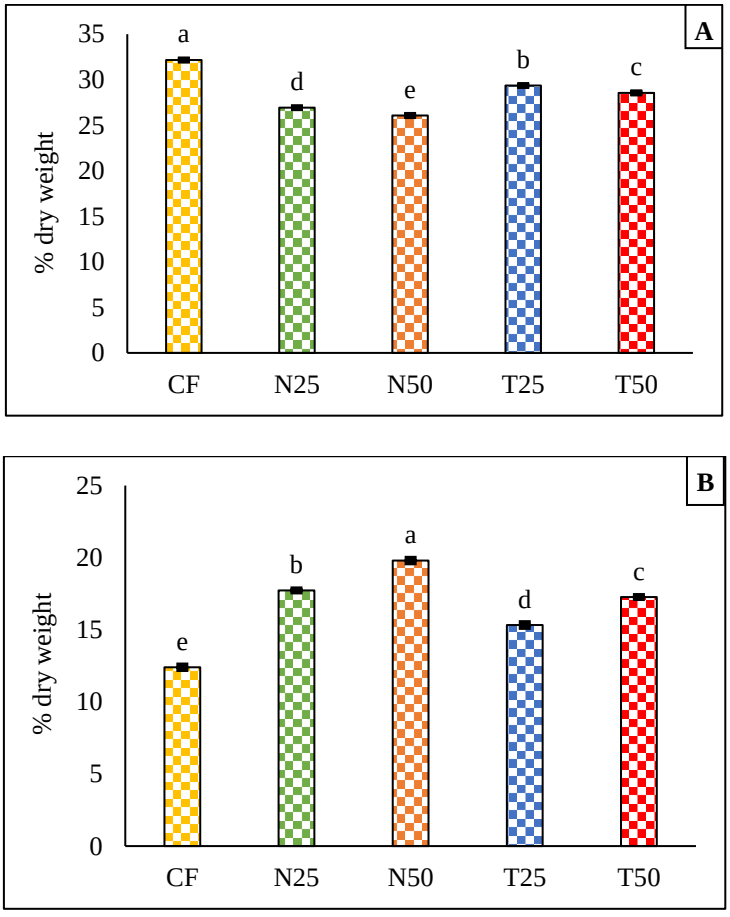
Treatment	Physical parameters			Chemical parameters		
	DO (mg/L)	pH	Temperature (^{0}C)	TAN (mg/L)	NO_2-N (mg/L)	SRP (mg/L)
CF	6.60 ± 0.03^{a}	8.54 ± 0.05^{a}	27.64 ± 0.07^{a}	0.66 ± 0.01^{a}	0.51 ± 0.01^{a}	0.15 ± 0.00^{a}
N25	6.58 ± 0.06^{a}	8.50 ± 0.05^{a}	27.65 ± 0.09^{a}	0.54 ± 0.00^{c}	0.44 ± 0.00^{c}	0.10 ± 0.00^{d}
N50	6.55 ± 0.05^{a}	8.44 ± 0.07^{a}	27.65 ± 0.09^{a}	0.59 ± 0.00^{b}	0.47 ± 0.00^{b}	0.13 ± 0.00^{b}
T25	6.55 ± 0.03^{a}	8.42 ± 0.05^{a}	27.80 ± 0.09^{a}	0.46 ± 0.00^{d}	0.42 ± 0.01^{d}	0.10 ± 0.00^{d}
T50	6.55 ± 0.04^{a}	8.42 ± 0.06^{a}	27.81 ± 0.12^{a}	0.54 ± 0.00^{c}	0.42 ± 0.01^{d}	0.11 ± 0.00^{c}

Mean ± SE (standard error) along with different small uppercase letters of chemical parameters within same column are statistically significant ($p < 0.05$); on the contrary same small uppercase letters of physical parameters within each column defining no significance ($p > 0.05$) which was determined by the multiple range test of Duncan.

4.3 Nutritional Composition of Nile Tilapia

4.3.1 Proximate Composition of Nile Tilapia Whole Fish

After the end of feeding trial, the protein, lipid and carbohydrate percentages of Nile tilapia fry of each treatment were determined by chemical analysis process and significant differences ($p < 0.05$) of whole fish muscle sample from individual treatment were documented (Figure 4). The observed values for protein, lipid, carbohydrate was ranged from 26.07 % to 32.16 % (Figure 4A), 12.4% to 19.8% (Figure 4B) and 9.59% to 12.78% (Figure 4C), consequently. In this study the highest protein, lipid and carbohydrate nutrient value was documented in CF (32.16±0.06%), N50 (19.8±0.12%) and T50 (12.78±0.02%) accordingly. The lowest values of protein achieved in N50 (26.07±0.06%), lipid in CF (12.4±0.12%) and carbohydrate in CF (9.59±0.02%) treatment consequently.



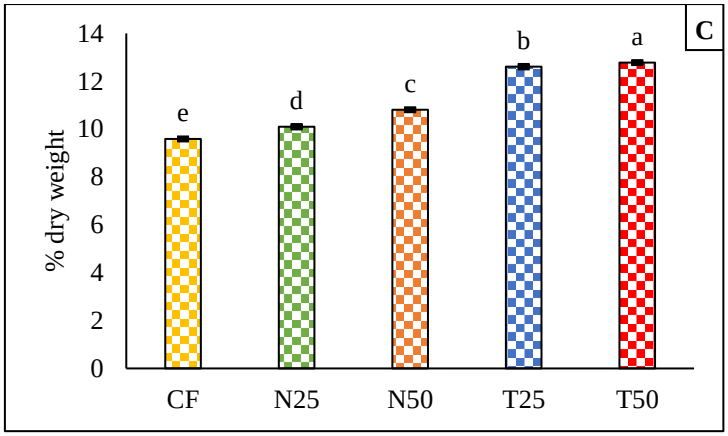


Figure 4: Proximate composition of Nile tilapia whole fish fed with *Nannochloropsis* sp. and *Tetraselmis* microalgae. A) Protein; B) Lipid and C) Carbohydrate. Values (n=3) are mean ± SE (standard error).

4.3.2 Fatty Acid Composition of Nile Tilapia Whole Fish

The result of percent total fatty acid composition of whole Nile tilapia fish tissue is presented in the following Table 5. The observed values indicating that percent of total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) are significantly affected ($p < 0.05$) by dietary microalgae and commercial feed fed diet.

Nile tilapia fish fed with commercial feed obtained the lowest C10:0, C16:0, C17:0, C21:0, C22:0, C23:0 and C24:0 percentage of saturated fatty acids in comparison to treatment N25, N50, T25 and T50, although lowest percentage of total SFA was observed in T50 (32.87 ± 0.01 %) and highest value recorded in N25 (35.49 ± 0.09%) treatment. In case of total MUFA, 25% *Nannochloropsis* sp.-based diet exhibited the lowest total MUFA. In regards, highest percentage of PUFA, eicosapentaenoic acid (EPA) C20:5n-3 was observed in N50 (22.42 ± 0.02%) treatment. Elevated concentration of *Nannochloropsis* sp. in fish diet has significantly increased the C20:5n-3 content in fish tissue compared to other treatment while in case of other PUFAs elevated concentration of microalgae did not significantly increase the PUFA content numerically. However, highest total n-3 PUFA and n-6 PUFA content was consequently found in CF (32.47 ± 0.14%) and T25 (23.78 ± 0.01%) treatment although highest total PUFA was recorded in T50 (52.02 ± 0.06%) treatment in comparison to other treatment.

Table 5 Fatty acid (% of total fatty acid) composition of Nile tilapia whole fish.

Carbon	Fatty acid	CF	N25	N50	T25	T50
C8:0	Octanoic acid	1.27 ± 0.01^{b}	1.48 ± 0.06^{a}	1.56 ± 0.04^{a}	1.22 ± 0.01^{b}	1.29 ± 0.01^{b}
C10:0	Decanoic acid	0.65 ± 0.02^{b}	0.92 ± 0.02^{a}	0.98 ± 0.07^{a}	0.68 ± 0.02^{b}	0.68 ± 0.01^{b}
C12:0	Lauric acid	4.72 ± 0.07^{a}	3.56 ± 0.03^{d}	3.39 ± 0.04^{d}	4.50 ± 0.08^{b}	4.22 ± 0.03^{c}
C13:0	Tridecanoic acid	0.47 ± 0.02^{a}	0.25 ± 0.03^{c}	0.35 ± 0.01^{b}	0.12 ± 0.01^{d}	0.15 ± 0.01^{d}
C14:0	Myristic acid	5.29 ± 0.04^{c}	5.76 ± 0.05^{a}	5.61 ± 0.02^{b}	5.37 ± 0.05^{c}	5.03 ± 0.03^{d}
C16:0	Palmitic acid	15.35 ± 0.02^{b}	16.47 ± 0.05^{a}	15.76 ± 0.06^{a}	16.79 ± 0.13^{b}	15.85 ± 0.09^{b}
C17:0	Heptadecanoic acid	0.12 ± 0.01^{d}	0.27 ± 0.01^{b}	0.31 ± 0.03^{c}	0.17 ± 0.02^{a}	0.23 ± 0.00^{c}
C18:0	Stearic acid	3.44 ± 0.11^{a}	2.94 ± 0.04^{b}	3.08 ± 0.01^{b}	2.54 ± 0.07^{c}	2.47 ± 0.04^{c}
C20:0	Arachidic acid	0.67 ± 0.01^{a}	0.65 ± 0.01^{ab}	0.69 ± 0.03^{a}	0.54 ± 0.03^{c}	0.58 ± 0.01^{b}
C21:0	Heneicosanoic acid	0.11 ± 0.00^{d}	0.52 ± 0.05^{a}	0.57 ± 0.02^{a}	0.22 ± 0.01^{c}	0.34 ± 0.02^{b}
C22:0	Behenic acid	0.36 ± 0.02^{c}	1.10 ± 0.04^{a}	1.18 ± 0.05^{a}	0.68 ± 0.02^{b}	0.79 ± 0.01^{b}
C23:0	Tricosanoic acid	0.06 ± 0.01^{d}	0.22 ± 0.01^{b}	0.28 ± 0.03^{a}	0.10 ± 0.01^{d}	0.16 ± 0.01^{e}
C24:0	Lignoceric acid	0.66 ± 0.04^{d}	1.33 ± 0.01^{a}	1.34 ± 0.01^{a}	0.89 ± 0.05^{c}	1.08 ± 0.03^{b}
	∑ **SFA**	33.18 ± 0.03^{d}	35.49 ± 0.09^{a}	35.09 ± 0.12^{b}	33.80 ± 0.11^{c}	32.87 ± 0.01^{e}
C16:1	Palmitoleic acid	3.02 ± 0.08^{b}	3.58 ± 0.05^{a}	3.71 ± 0.04^{a}	2.90 ± 0.07^{b}	2.98 ± 0.02^{b}
C18:1	Oleic acid	7.17 ± 0.11^{c}	7.47 ± 0.03^{b}	7.35 ± 0.04^{b}	9.91 ± 0.05^{a}	9.75 ± 0.03^{a}
C20:1	cis-11-Eicosenoic acid	0.41 ± 0.06^{b}	0.67 ± 0.06^{a}	0.76 ± 0.02^{a}	0.42 ± 0.01^{b}	0.50 ± 0.05^{b}
C22:1	Erucic acid	6.86 ± 0.06^{a}	2.23 ± 0.05^{c}	2.47 ± 0.03^{b}	1.48 ± 0.05^{e}	1.78 ± 0.05^{d}
C24:1	Nervonic acid	0.05 ± 0.00^{d}	0.13 ± 0.00^{a}	0.16 ± 0.02^{a}	0.09 ± 0.01^{c}	0.10 ± 0.00^{b}
	∑ **MUFA**	17.52 ± 0.03^{a}	14.09 ± 0.08^{e}	14.43 ± 0.09^{d}	14.80 ± 0.07^{c}	15.11 ± 0.06^{b}
C18:2n-6	Linoleic acid	10.84 ± 0.05^{d}	11.43 ± 0.04^{c}	11.50 ± 0.07^{c}	18.74 ± 0.05^{a}	17.62 ± 0.05^{b}
C20:3n-6	Eicosatrienoic acid	5.45 ± 0.04^{a}	4.91 ± 0.00^{b}	4.73 ± 0.07^{c}	3.75 ± 0.04^{e}	4.31 ± 0.02^{d}
C20:4n-6	Arachidonic acid	0.55 ± 0.05^{c}	1.79 ± 0.03^{b}	1.20 ± 0.01^{a}	1.30 ± 0.10^{c}	1.64 ± 0.01^{b}
C18:3n-3	Linolenic acid	16.48 ± 0.12^{a}	8.37 ± 0.15^{c}	8.26 ± 0.05^{c}	10.18 ± 0.04^{b}	10.16 ± 0.02^{b}
C20:5n-3	Eicosapentanoic acid	15.12 ± 0.06^{d}	22.32 ± 0.01^{a}	22.42 ± 0.02^{a}	16.22 ± 0.05^{c}	16.65 ± 0.05^{b}
C22:5n-3	Docosapentaenoic acid	0.57 ± 0.03^{c}	1.49 ± 0.03^{a}	1.42 ± 0.10^{a}	0.91 ± 0.07^{b}	1.17 ± 0.01^{b}
C22:6n-3	Docosahexaenoic acid	0.29 ± 0.01^{b}	0.11 ± 0.01^{d}	0.15 ± 0.00^{c}	0.31 ± 0.00^{b}	0.47 ± 0.01^{a}
	∑ **PUFA**	49.30 ± 0.00^{d}	50.43 ± 0.17^{c}	50.48 ± 0.21^{c}	51.40 ± 0.04^{b}	52.02 ± 0.06^{a}
	∑ **n-3**	32.47 ± 0.14^{a}	32.29 ± 0.10^{a}	32.25 ± 0.07^{a}	27.62 ± 0.03^{c}	28.45 ± 0.03^{b}
	∑ **n-6**	16.83 ± 0.14^{c}	18.13 ± 0.07^{b}	18.23 ± 0.15^{b}	23.78 ± 0.01^{a}	23.57 ± 0.03^{a}
	∑ **n-3/n-6**	1.93 ± 0.02^{a}	1.78 ± 0.00^{b}	1.77 ± 0.01^{b}	1.16 ± 0.00^{d}	1.21 ± 0.00^{c}

Values (n=2) with different small uppercase within individual column are as mean ± SE (standard error) and showing significancy ($p < 0.05$) followed by Duncan multiple range test.

4.4 Total Carotenoid Concentration of Nile Tilapia Fish Tissue

In this study, microalgae were proven to be an efficient color enhancer and values of five treatment CF, N25, N50, T25 and T50 varied significantly ($p < 0.05$). Between the *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae fed diet *Nannochloropsis* sp. exhibited amazing color enhancing performance in Nile tilapia fish tissue in comparison to *Tetraselmis* sp. Among 380 nm, 450 nm, 470 nm and 500 nm wave length, highest absorption was recorded in 450 nm wavelength. As a result, absorption values in 450 nm wavelength were used for the determination of total carotenoid concentration. The highest concentration of total carotenoid content in wet weight was documented in N50 (3.27 ± 0.04) µg/g and the lowest concentration was recorded in CF (0.87 ± 0.03) µg/g (Figure 5). Correspondingly, the total carotenoid concentration in T50, N25 and T25 treatment also showed significantly ($p < 0.05$) higher values compared to CF (Figure 5).

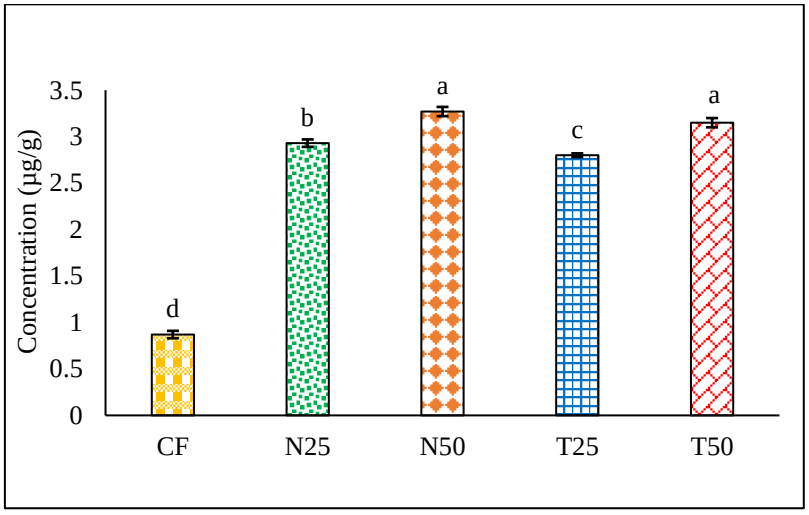


Figure 5: Total carotenoid concentration of Nile tilapia fish tissue fed with *Nannochloropsis* sp. and *Tetraselmis* microalgae. Values (n=3) are mean ± SE (standard error).

4.5 The Effect of Microalgae Fed Diet on Hemato-biochemical Index of Nile Tilapia

4.5.1 Blood Hematology of Nile Tilapia

Hematological assessments are common practices in which the quality of fish and other existing organisms living in temporal places is determined. In this study, effect of microalgae fed diet on fish hematology was recorded with different nutritional state acquired through different inclusion rate of *Nannochloropsis* sp. and *Tetraselmis* sp. RBC, Hb, Hct, WBC, LYM and PLT are the essential components of blood hematology which can act like biomarkers. Because significant changes of these blood parameters can indicate the physiological changes of fish also. The results of Table 6 indicating that *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae could bring significant change in the hematological parameters of Nile tilapia and all the results varied significantly among the five treatment ($p < 0.05$). Significant higher values of RBC, Hb, Hct and WBC were recorded in T25 ($1.74 \pm 0.021 \times 10^6$/µL), ($9.6 \pm 0.11$ g/dl), (32.6 ± 0.14 %) and ($26.3 \pm 0.27 \times 10^3$/µL) respectively, compared to the control. Lower level of these parameters was recorded in CF which are correspondingly, ($1.46 \pm 0.014 \times 10^6$/µL), ($6.8 \pm 0.12$ g/dl), (23.3 ± 0.17%) and ($18.4 \pm 0.29 \times 10^3$/µL). Lower level of RBC in CF automatically lowered the Hb and Hct of Nile tilapia blood (Table 6). In contrast, significantly higher level of LYM and PLT was recorded in CF (58.6 ± 0.31 %) and ($66.3 \pm 0.05 \times 10^3$/µL). However, lowest LYM and PLT was found accordingly, in T25 (47.3 ± 0.2%) and N50 ($28.6 \pm 0.12 \times 10^3$/µL), followed by T50 ($37.4 \pm 0.14 \times 10^3$/µL) treatment (Table 6) (Amira et.al., 2021) after the 8-week experiment.

Table 6 Impact of *Nannochloropsis* sp. and *Tetraselmis* sp. supplemented diet on hematological parameters of Nile tilapia (Amira et.al., 2021).

Parameters	Unit	Treatment				
		CF	N25	N50	T25	T50
RBC	$\times 10^6$/µL	1.46 ± 0.014^d	1.67 ± 0.005^b	1.61 ± 0.008^c	1.74 ± 0.021^a	1.72 ± 0.011^a
Hb	g/dl	6.8 ± 0.12^e	8.3 ± 0.15^c	7.4 ± 0.12^d	9.6 ± 0.11^a	8.7 ± 0.11^b
Hct	%	23.3 ± 0.17^e	28.2 ± 0.14^c	27.2 ± 0.14^d	32.6 ± 0.14^a	30.5 ± 0.14^b
WBC	$\times 10^3$/µL	18.4 ± 0.29^e	21.4 ± 0.24^c	19.3 ± 0.2^d	26.3 ± 0.27^a	23.4 ± 0.23^b
LYM	%	58.6 ± 0.31^a	52.1 ± 0.18^c	56.4 ± 0.26^b	47.3 ± 0.2^e	48.2 ± 0.25^d
PLT	$\times 10^3$/µL	66.3 ± 0.05^a	45.5 ± 0.08^b	28.6 ± 0.12^e	43.1 ± 0.08^c	37.4 ± 0.14^d

Mean ± SE (standard error) along with different small letters (uppercase) within each column are showing significancy ($p < 0.05$) by following multiple range test of Duncan.

4.5.2 Blood Serum Biochemical Parameters of Nile Tilapia

Changes in blood serum component helps to identify certain functional disorders of body organ and health status of fish such as, total protein, albumin, globulin and A/G ratio detects blood nutritional status. Through triglyceride and cholesterol measurement, fat composition stored in blood can be identified, blood glucose change indicates the liver function, urea and blood urea nitrogen represents kidney disorders if present in fish. Analysis of these serum parameters defines the overall immunity level of fish. This study identified significant variation in overall blood serum profile of Nile tilapia fish as an effect of *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae inclusion in diet.

In the current study, total protein (Figure 6A), albumin (Figure 6B), globulin (Figure 6C) and A/G ratio (Figure 6D) of blood serum of Nile tilapia fish were illustrated in Figure 6 by comparing it with control. Significant variation in all the values ($p < 0.05$) was noticed. Replacement of fish meal with microalgae showed lower response in total protein, albumin and globulin concentration elevation in T25, T50, N25 and N50 treatment in comparison to CF treatment. N50 obtained the lowest level of total protein (3.9 ± 0.02 g/dl), albumin (1.9 ± 0.01 g/dl) and globulin (2.0 ± 0.02 g/dl). In contrast, CF achieved the highest level of total protein (6.09 ± 0.03 g/dl), albumin (2.47 ± 0.02 g/dl) and globulin (3.62 ± 0.02 g/dl) (Amira et.al., 2021). No significant difference ($p < 0.05$) was observed in case of albumin in T25 and T50 (Figure 6B). Meanwhile, N50 showed the highest A/G ratio (0.94 ± 0.02). Furthermore, T25 showed almost similar result of A/G ratio with CF and T50 (Figure 6D).

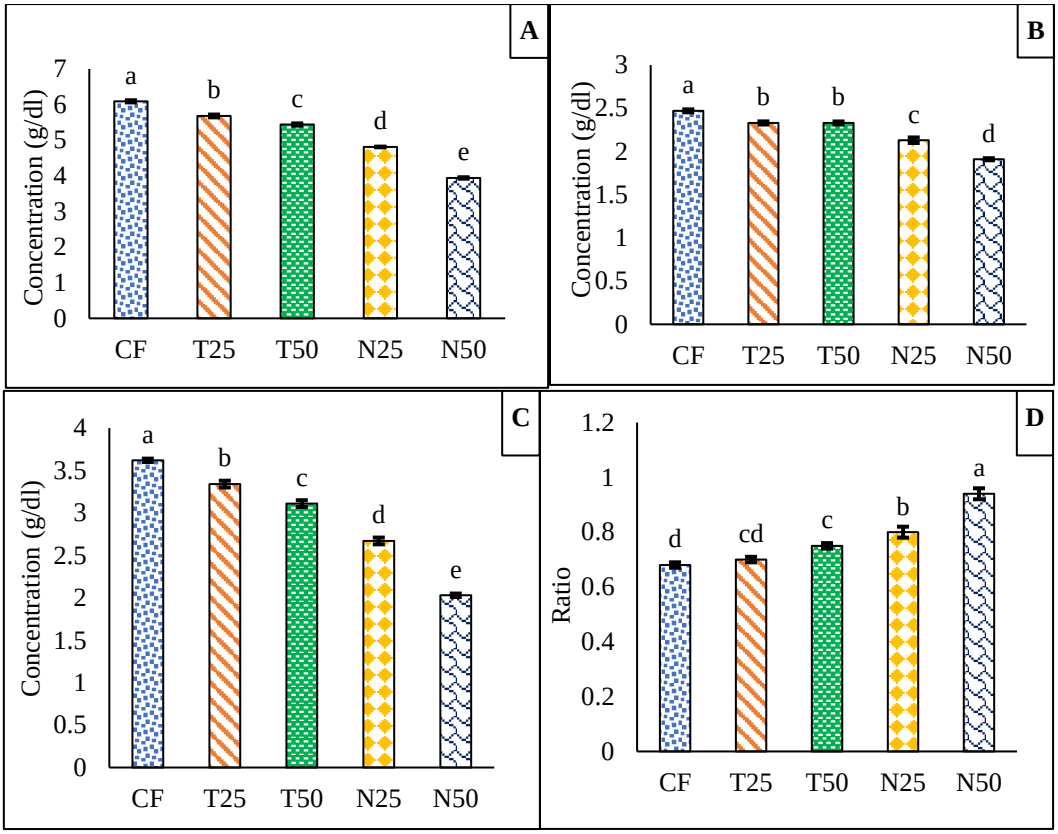


Figure 6: Blood serum A) total protein; B) albumin; C) globulin and D) A/G (albumin/globulin) ratio of Nile tilapia fish fed with *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae. Values (n=3) are mean ± SE (standard error).

In figure 7 significant changes in fat component triglyceride (Figure 7A) and cholesterol (Figure 7B) is illustrated. In this regard, microalgae fed treatment T25, T50, N25 and N50 showed significant ($p < 0.05$) declining impact towards those blood serum components in contrast to control. However, high level of triglyceride and cholesterol content in blood serum is not desirable for fish health. In addition, N50 lowered the triglyceride (194.1 ± 0.27 mg/dl) and cholesterol (204.5 ± 0.35 mg/dl) value by showing the maximum positive response compared to CF and CF obtained the highest triglyceride (213.6 ± 0.27 mg/dl) and cholesterol (250.3 ± 0.2 mg/dl) concentration in blood (Amira et. al., 2021).

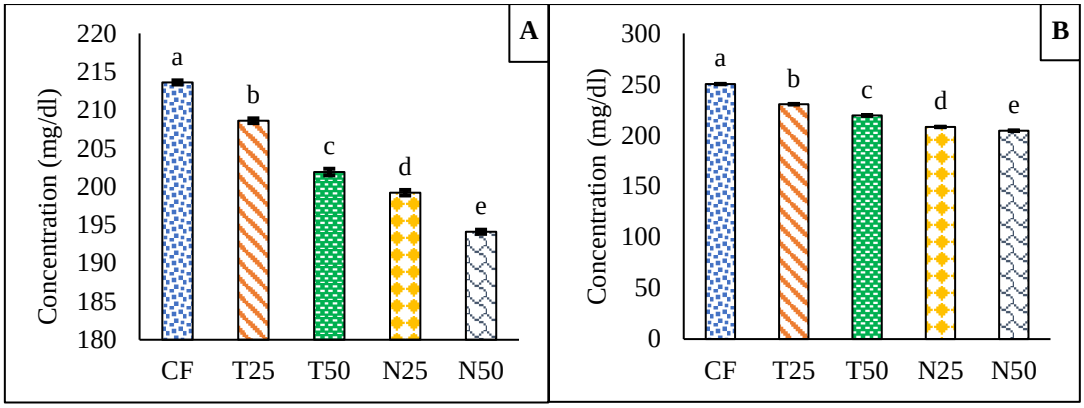


Figure 7: Blood serum A) triglyceride; B) cholesterol concentration of Nile tilapia fish fed with *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae. Values (n=3) are mean ± SE (standard error).

Significant variation in blood glucose concentration was depicted in Figure 8. Microalgae fed treatment T25, T50, N25 and N50 showed significant ($p < 0.05$) lowering impact of blood glucose in comparison to CF. In response to stress indication CF achieved the highest (79.2 ± 0.28 mg/dl) concentration of blood glucose and T25 obtained the lowest (67.5 ± 0.08 mg/dl) concentration of blood glucose (Amira et.al., 2021).

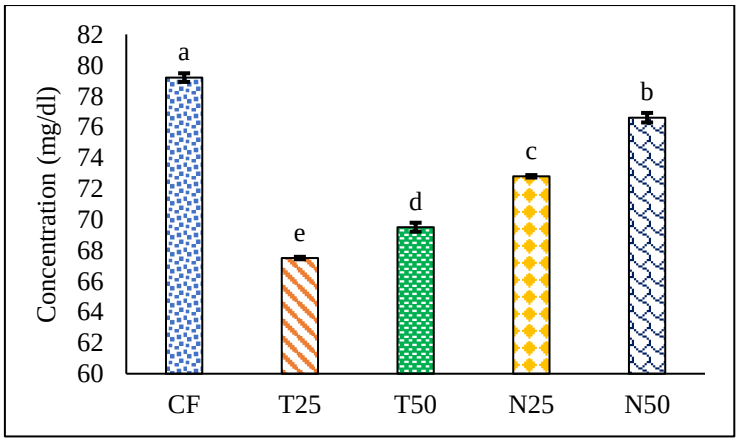


Figure 8: Blood serum glucose concentration of Nile tilapia fish fed with *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae. Values (n=3) are mean ± SE (standard error).

Urea and BUN are regarded as waste product of fish body which circulates through blood. It is noteworthy that, high concentration of urea led to a higher concentration of BUN in blood serum. That's why, high concentration is not desirable for fish health. Figure 9 depicted the significant variation ($p < 0.05$) in urea (Figure 9A) and BUN

(Figure 9B) values with similar outcomes comparing with control. Lowest concentration of urea and BUN was noted in N50, (23.8 ± 0.18 mg/dl) and (11.1 ± 0.08 mg/dl) respectively. Correspondingly, CF exhibited the highest concentration of urea (30.9 ± 0.47 mg/dl) (Figure 9A) and BUN (14.4 ± 0.26 mg/dl) (Figure 9B) (Amira et.al., 2021). However, almost similar result was observed for N25 with, T50 and N50 for both of the parameters.

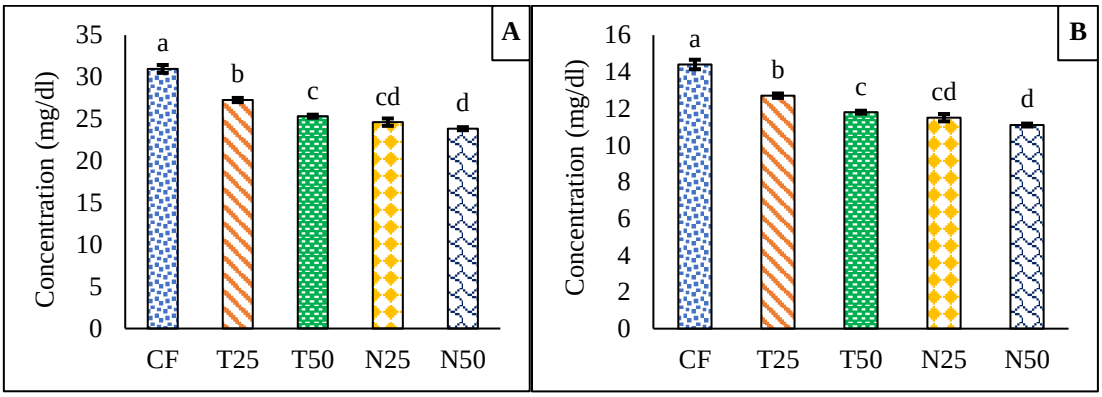


Figure 9: Blood serum A) urea and B) BUN concentration of Nile tilapia fish fed with *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae. Values (n=3) are mean ± SE (standard error).

Chapter-5: Discussion

5.1 Growth Performance of Nile Tilapia

Expected outcomes were not obtained in case of growth study in this research (Amira et.al., 2021). Feeding trial conducted using microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. exhibited poor growth and feed utilization performance (Table 3) compared to control treatment (Amira et.al., 2021). In control treatment fish meal was the sole protein source and indicated that Nile tilapia fish responded very well towards fish meal compared to microalgal meal. Fish meal alone is a high source of protein in diet for Nile tilapia. However, in this research *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae alone with the replacement of fish meal could not performed well. In between the comparison of two microalgae group N25 and N50 showed very poor growth performance than T25 and T50.

Cultured fish performance is heavily impacted by some factors such as fish behavior, stocking density, feed quality, daily ration size and frequency of feeding as well as water temperature (Alemayehu and Getahun, 2017). FCR is the quantity of feed needed to produce one kilogram of fish and it's regarded that lower FCR values indicate better feed utilization rate. In Africa the FCR values was recorded between 1.4 and 2.5 in tilapia cage aquaculture systems (Ofori et al., 2009). In this study relatively higher FCR values was obtained. Similar results of this study for FCR were also noticed in the research of Olvera-Novoa et al. (1998). In this study higher condition factor was noticed in CF. Condition factor of fish is the indication of basic well-being of fish health. It demonstrates the changes of fish weight according to body length and it's also used to explain the fish physical condition during specific culture duration (Ighwela et al., 2011). Although microalgae are rich in vitamins, minerals, carotenoids, essential amino acids and fatty acids (PUFA) this could not help to increase the growth of Nile tilapia fish (Amira et. al., 2021). Higher proportion of microalgae also contain more fibre content which is not easily digestible (Sarker et al., 2018) in fish body and responsible to create palatability problem. In this sense, the dietary quality of microalgae protein might be less high than that of fish meal (Amira et.al., 2021).

In this research the lowest growth in N50 was may be due to two major issues; i) in the cell wall of *Nannochloropsis oculata,* indigestible high concentration of composite non-starch polysaccharides has found (Domozych et. al., 2012). Surprisingly, high concentration of polysaccharide complex was discovered in the *Nannochloropsis* sp.

cell walls (Scholz et. al., 2014). Due to lowering impact of the nutrient absorption in fish intestinal tract the composite polysaccharides that are non-starch (for example, cellulose, gums, pectins and hemicellulose) regarded as undesirable for feed in aquaculture (Norambuena et. al., 2015). ii) large quantities of anti-nutrients comprised by high concentration of *Nannochloropsis oculata* microalgae which was responsible for preclusion of trypsin by prohibiting the nutrient to digest thus, modified the morphology and physical composition of the fish intestines (Sarker et al., 2018). Nevertheless, these problems might be resolved during feeding experiment if replacement could be done from all the feed ingredients in much lower percentage thus could able to reduce antinutritional and palatability problems. Findings of Walker and Berlinsky (2011), was in line with this study. They elucidated that when feed was formulated using 15% and 30% (dry weight basis) microalgae *Isochrysis* sp. and *Nannochloropsis* sp. as a replacement of fish meal it gradually lowered the feed consumption and as a result growth rate of Atlantic cod, *Gadus morhua* declined significantly. Sarker et al. (2018) also found similar result and stated that, with the increasing percentage of microalgae *Nannochloropsis oculata* at dietary level of 33%, 66% and 100% in fish diet could significantly reduce the growth performance of Nile tilapia compared to control diet.

Dietary microalgae have also been seen to have a negative impact on fish health, reported by other researchers. For instance, in comparison with the reference diet of fish which was based on fish meal, 10 percent (dry-weight basis diet) freeze-dried meals derived from *Tetraselmis chuii* and *Phaeodactylum tricornutum*, separately or in addition with *Bacillus subtilis* (10^7 CFU / g) probiotic, in *Sparus aurata*, decreased variety of bacteria and disturbed bowel morphologies (Cerezuela et al., 2012); at the same time insignificant inflammation of the digestive area was caused by 10 percent inclusion of freeze-dried *Navicula* sp. in dry weight diet. Buentello et al. (2015), indicated that these was may be due to some meals of microalgae which contains antinutritional components, for example protease inhibitors and oligosaccharides present in soybean meal like basal source of protein, it prompted related responses in different fish species, may explain the evidence of disturbed fish digestive area and decreased palatability. Conversely, contradictory results were found in some earlier studies and evidence were reported by different researchers that, microalgae could successfully enhance the growth performance of different fish species. Patterson and Gatlin (2013) reported that up to 10% of crude protein replacement from fish meal and

soybean meal using *Nannochloropsis salina* could be possible without causing any significant negative impact on red drum (*Sciaenops ocellatus*) juveniles growth performance. Another study conducted by Kiron et al. (2012) found that either 5 or 10% replacement of dietary protein using whole and lipid-extracted algal meals from fish meal did not have a substantial impact on Atlantic salmon (*Salmo salar*) weight gain compared to those fed on control diets. Two algal products (*Tetraselmis* and *Nanofrustulum*) could substitute 25-40% of the protein level in diets of *Litopenaeus vannamei* and *Cyprinus carpio*, stated by these authors. Discrepancies between findings of this research and other findings may be attributed to a variety of reasons, including, i) source, species and culture characteristics of *Nannochloropsis* sp. and *Tetraselmis* sp. ii) rearing condition of fish iii) the research plan and arrangement iv) dissimilar fish species containing diverse structural assemblies of the digestive area and v) different feed formulation and composition. Nevertheless, further investigation is required to mitigate all sort of discrepancies.

The findings of survival rate of this research were impressive (Table 3) and accomplished the expected result. Survival rate of fish fed on *Tetraselmis* sp. and *Nannochloropsis* sp. were varied significantly among the treatments. Microalgae fed treatments T25, T50, N25 and N50 exhibited higher survival in comparison to the control diets and increased survival rate from (61.11 to 92.59%). However, highest positive response came from T25 treatment. This result suggests that *Tetraselmis* sp. and *Nannochloropsis* sp. microalgae was habituated very well as feed of Nile tilapia fry and did not negatively impact the fish health. Likewise, in the study of Patterson and Gatlin (2013) 5% supplementation of microalgae *Nannochloropsis salina* significantly increased the survival of red drum (*Sciaenops ocellatus*) juvenile. In a similar way, up to 92.8% survival rate was achieved in a diet of Nile tilapia prepared with a mixture of microalgae *Spirulina platensis* and *Chlorella vulgaris* which was revealed in the research of Hossain et al. (2017). Furthermore, in the study of Mukherjee et al. (2019) confirmed that, during mixed algal meal feeding, low mortality rate of goldfish and carps have been obtained due to least stress enzyme aggregation in fish body. High survivality was achieved in this research might be due to presence of some bioactive compounds in *Tetraselmis* sp. and *Nannochloropsis* sp. such as, carotenoids, vitamin E, terpenes and phenolic complexes comprising anthelmintic, cytostatic, antioxidant, antifungal, antiviral and antibacterial actions which was absent in fish meal (Amira

et.al., 2021) and thus, helped to boost the immunity (Brown et. al., 1999; Newman et al., 2003) of Nile tilapia fry when fed diet containing microalgae except control.

5.2 Water Quality

The environment in which fish lives need to be well suited for fish health. In this sense optimum water quality is required for fish culture (Amira et.al., 2021). In this study the values obtained for physical parameters: DO, temperature, pH was within the threshold level (Table 4). DeLong et al. (2009) stated that, best growth performance will be achieved if tilapia is reared in temperature between (27 to 29°C), DO between (5.0 to 7.5 mg/L) and pH between (6-9). The values of DO, pH and temperature showed in Table 4 are within these range and did not show significant changes among all the treatments.

Higher concentration of ammonia, nitrite and soluble reactive phosphorus is not desirable in aquaculture. As high ammonia further converts into nitrite can be toxic for fish health. In this study microalgae fed treatment showed optimum performance and TAN, NO_2-N, SRP values of all the microalgae fed treatments are in expectable range compared to control (Table 4).

TAN is the most important water quality parameter which can critically affect fish health. TAN concentration was between (0.46 to 0.66 mg/L) and varied significantly during the experiment (Table 4). Though Boyd (1998) recommended to maintain ammonia concentration below 0.1 mg/L for aquaculture. On the other hand, Caldini et al. (2015) provided an optimal range of TAN between (0.17 to 3.87 mg/L) for Nile tilapia culture. The range of TAN of this study is in agreement with the study of Caldini et al. (2015). DeLong et al. (2009) also suggested that below 1 mg/L unionized ammonia concentration is not harmful for rearing tilapia. NO_2-N concentration also varied significantly among all the treatment and ranged between (0.42 to 0.51 mg/L) (Table 4) which is higher than the optimum concentration < 0.3 mg/L recommended for aquaculture by Boyd (1998). However, nitrite nitrogen concentration never exceeded the undesirable level recommended to avoid by DeLong et al. (2009) which is higher than 5 mg/L. SRP concentration was ranged between (0.10 to 0.15 mg/L) during the culture period and also significantly varied (Table 4) among the five treatments. However, (0.005 to 0.2 mg/L) concentration is the acceptable limit of phosphorus concentration in water for fish culture reported by Boyd (1998). High concentration of soluble reactive phosphorus indicates pollution of culture condition

yet the values of phosphorus concentration of this study had never exceeded the acceptable level during the culture period recommended by this author.

Feed which fed, uneaten feed and feces derived from fish during the experiment are the main sources of TAN, NO_2-N and SRP in this study. High level of ammonia and nitrite in culture water can cause biochemical changes of fish health by affecting oxygen transportation in blood. In nitrite exposed fish, extreme reduction of blood hemoglobin, hematocrit and red blood cell were noticed by previous researchers and significant low level of hemoglobin content in blood may indicate methaemoglobinaemia (Jensen, 2003) thus ultimately lowers the fish survivality. Although the TAN, NO_2-N and SRP concentration were within optimal range provided by previous researchers but highest TAN, NO_2-N and SRP was noticed in the control treatment which could affect the survivality (Table 4) of Nile tilapia in this research. In contrast, use of microalgae as a replacement of conventional feed ingredients is very much useful for improving culture water which was noticed in the treatment of T25, T50, N25 and N50 when fed *Tetraselmis* sp. and *Nannochloropsis* sp. microalgae containing diet during the experiment. This was may be due to the water stabilizing capability of microalgae. It was stated by previous researchers that, when microalgae are used in fish diet, the uneaten feed remains in culture water contains algal cells which can survive in rearing water of cultivated fish without disrupting water quality or causing eutrophication in that environment, as microalgae retains nutrients like, carbon, phosphorus and nitrogen remains from uneaten feed (Yang et al., 2021). Hence, cells of microalgae may start developing in the tank from the residual feed. In this experiment, gradual rise of the slight green color was noticed during the culture period in the culture tank especially in the treatment of T25, followed by T50. It was may be due to the contribution of rest of the microalgae remained in the culture tank which absorbed the available nutrients of water. This finding is in line with the finding of Chen et al. (2012) who confirmed that *Tetraselmis chuii* has the maximum TAN accumulation capacity resulting in lesser nutrient in cultivation relative to the other studied microalgae organisms. Thus, it is reconciled in this study that, microalgae treated tank could save time and energy by supporting minimal water exchange and providing good water quality.

5.3 Nutritional Profile of Nile Tilapia

5.3.1 Proximate Composition of Nile Tilapia Whole Fish

A microalgal diet's nutritional value is directly proportional to its capacity to offer needed macro- and micronutrients to the intended animal consumer and fish exhibit its nutritional status in muscle tissue according to the nutrition provided into its diet. In the current study the results of proximate composition of protein, lipid and carbohydrate (Figure 4A-4C) of Nile tilapia is significantly affected by different diet composition. One of the primary reasons for considering microalgae as a nontraditional source of protein is their high protein content (Becker, 2007) but in the current study among the fish meal, *Nannochloropsis* sp. and *Tetraselmis* sp.-based diet, treatment with commercial fish meal-based diet has shown better protein percentage (Figure 4A) and this might be due to presence of high fibre content in high percentage in microalgae which has reduced the digestibility of microalgae-based diet in fish body (Sarker et al., 2018). Sørensen et. al. (2021) also found similar results in his study and confirmed that fish meal-based diet has shown higher protein percentage in whole body composition in Atlantic Salmon (*Salmo salar*) fish than *Nannochloropsis oceanica* and *Tetraselmis* sp. fed diet. Tibbetts (2018) documented that, *Nannochloropsis* has a protein composition that ranges from 18 to 48 percent and lipid composition ranges from 2 to 68 percent besides *Tetraselmis* contain protein and lipid ranges from 27 to 54 percent and from 3 to 45 percent correspondingly. Between *Nannochloropsis* sp. and *Tetraselmis* sp. comparatively higher protein nutrition obtained in T25 and the highest lipid content among all the treatment was recorded in N50 treatment (Figure 4B). Carbohydrates are the cheapest energy sources for fish diets and gradual increase in carbohydrate content than control was noticed in this study (Figure 4C). Carbohydrates are added in aquaculture diets, despite the fact that they are not required, to minimize feed costs and for their binding action. The study of Radhakrishnan et al. (2015) is in agreement with the current study and documented that 50% replacement of fish meal with *Chlorella vulgaris* microalgae can increase the carbohydrate content in *Macrobrachium rosenbergii* post larvae. For economical production of healthy and high-quality product good nutrition is essential and in this regard this study suggests to reduce the inclusion of microalgae (Sarker et.al., 2020) to get better protein nutrition in whole fish composition.

5.3.2 Fatty Acid Composition of Nile Tilapia Whole Fish

Saturated, mono unsaturated and poly unsaturated fatty acids (PUFAs) are three kinds of fatty acids. Omega-3 and omega-6 series are two significant groups of PUFAs regarded as the most important bioactive molecules for living species (Li et al., 2014). Several dietary components such as minerals, vital fatty acids of omega-3 and omega-6, and other useful nutrients are found abundant in microalgae (Tokusoglu and Ünal, 2001). Among PUFAs, notably for EPA and DHA, omega-3 fatty acids are universally acknowledged and proved to be beneficial (Siriwardhana et.al., 2012; Tocher, 2015) in prevention or treatment of a variety of illnesses of human beings which includes blinding retinal conditions, cardiac illness, diabetes, malignance and dementia (SanGiovanni and Chew, 2005; Macchia et al., 2013; Shahidi and Ambigaipalan, 2018). EPA and DHA are mostly found in fish (Tocher, 2015). Nevertheless, in aquaculture fish meal and fish oil is considered as unsustainable source to obtain the EPA and DHA in fish. The supply of fishmeal and fish oil will be insufficient to fulfill the demand because of the fast growth of aquaculture. To partially replace fish meal and fish oil alternatives such as soybean oil and soybean meal have frequently been employed because of their ease of harvesting (Wang et al., 2014) and tolerance to microbes. In this aspect microalgae also can be a potential substitute to mitigate the essential fatty acid supply demand.

In the current study, the addition of microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. in diet has shown a significant positive impact on the fatty acid profile of whole Nile tilapia fish (Table 5) and desired results are obtained in this study. According to the presented data the most abundant SFA, palmitic acid (C16:0); MUFA, oleic acid (C18:1) was found in T25 treatment. Essential PUFAs, like linoleic acid (18:2n-6), EPA (C20:5n-3) and DHA (C22:6n-3) were found dominant in T25, N50 and T50 treatment, respectively. The results of this study are similar to the previous research of Mohammadi et. al (2015) who found that palmitic acid, oleic acid, alpha-linoleic acid, EPA are consequently the most abundant SFA, MUFA, PUFA and highly unsaturated fatty acid (HUFA) in *Tetraselmis chuii* among all types of fatty acids. According to the study of Servel et al. (1994) and Schneider et al. (1995) the most abundant PUFA, EPA was found in the genus *Nannochloropsis* which was also confirmed in the study of Tonon et al. (2002). However, significant changes in n-3/n-6 ratio were observed in different treatment (Table 5). Higher n-3/n-6 ratio found in CF than other treatment.

This result is might be due to comparatively higher total n-3 PUFA and presence of lowest total n-6 PUFA in fish meal containing diet, although higher amount of n-6, C20:4n-6 was seen in fish meal fed treatment (Table 5). This result is similar to the findings of Gbadamosi and Lupatsch (2018) and found contradictory to the result of Chen et al. (2019). The maintenance of n-6 to n-3 PUFAs are regarded as important for human health for their physical development, homeostasis and psychological health (Simopoulos, 2011). In this experiment Nile tilapia fish received SFAs, MUFAs and essential PUFAs from *Nannochloropsis* sp. and *Tetraselmis* sp. inoculated diet.

Previous researchers like, Takeuchi et al. (2002) fed *Spirulina* sp. to Nile tilapia, Dos Santos et. al. (2019) fed *Schizochytrium* sp. to Nile tilapia, Hossain et. al. (2017) fed *Chlorella vulgaris* and *Spirulina platensis* to Nile tilapia, Gbadamosi and Lupatsch (2018) fed *Nannochloropsis salina* to Nile tilapia and found elevated level of PUFAs in fed fish. According to previous studies, both *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae are amazing source of some essential fatty acids, long chain PUFAs, especially EPA (Servel et. al., 1994; Schneider et.al., 1995; Meseck et. al., 2005; Zaki and Saad, 2010; Gbadamosi and Lupatsch, 2018) which is confirmed in the current research. In this regard, our data show that *Nannochloropsis* sp. and *Tetraselmis* sp. may be useful in enhancing the fatty acid profile of whole fish composition. As a result, for the improvement of the deposition of human health-facilitating fatty acids, the addition of *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae into fish diet is a smart strategy to boost the nutritional value of whole fish tissue.

5.4 Total Carotenoid Concentration of Nile Tilapia Fish Tissue

Fish skin color is an essential predictor of consumer acceptance, and carotenoids are responsible for fish pigmentation. Carotenoids must be supplemented in the diet of farmed species because fish cannot generate them on their own (Gupta et.al., 2007). It has been proposed that microalgae such as *Nannochloropsis* sp. and *Tetraselmis* sp. can supply the aquaculture sector with vital vitamins, pigments, and polyphenols (Brown et. al., 1999; Pataroa et.al., 2019; Kokkali et.al., 2020). Desired results were obtained in the current study. Carotenoids-rich *Nannochloropsis* sp. and *Tetraselmis* sp. (Di Lena et. al., 2018) show considerable significance as natural fish colorants and confirmed the presence of total carotenoids in Nile tilapia muscle tissue (Figure 5). Although gradual increase in total carotenoid concentration was noticed in both *Nannochloropsis* sp. and *Tetraselmis* sp. fed treatment than control however highest performance was exhibited

by N50 treatment. Carotenoids, are naturally occurring pigments that can serve as provitamin A and are found in microalgae at a level of 0.1-0.2 percent dry matter, are generated de novo predominantly by photosynthetic organisms (Becker, 1994). Though there are significant differences in the results of *Nannochloropsis* sp. and *Tetraselmis* sp. fed treatment similar results obtained in the study of Tulli et. al. (2012) and a change in skin pigmentation was detected after feeding *Tetraselmis suecica*-containing meals to juvenile sea bass. Greenish pigmentation was shown to be enhanced in fish fed diets including microalgae, similar to what was seen in *Carassius carassius* (Gouveia and Rema, 2005). Teimouri et al. (2013) also found that 10% addition of *Spirulina platensis* has resulted in maximum carotenoid deposition in the skin and fillet tissue of rainbow trout (*Oncorhynchus mykiss*) as a natural pigment source. According to research, it's found that eating a diet high in carotenoids can lower the incidence of free radical-related disorders including atherosclerosis and cancer (Gouveia et al., 2008).

5.5 Hemato-biochemical Index of Nile Tilapia

5.5.1 Blood Hematology of Nile Tilapia

In the current study, inclusion of *Nannochloropsis* sp. and *Tetraselmis* sp. in diet showed variation in the hematological parameters which were measured. *Nannochloropsis* sp. and *Tetraselmis* sp. in diet has positively influenced the RBC, Hb, Hct, WBC and significantly led to the rise in all parameters in comparison to control. T25 treatment, followed by T50 showed the highest positive response towards hematology (Table 6). The values of RBC, Hb, Hct, WBC, LYM and PLT of Nile tilapia are within reference range with the values provided by Hrubec et al. (2000) and Mauel et al. (2007) for tilapia fish (Amira et.al., 2021).

RBC is related to the oxygen carrying capacity of teleost fish species. Decreasing level of RBC in CF (Table 6) is indicating that, less oxygen binding molecule Hb is present in blood and restricted fish capacity to deliver the tissues with enough oxygen, resulted in physical activity decline. However, Hb loss is dangerous for oxygen transfer and any form of blood dyscrasia and erythrocyte degradation in fish exposed to toxicants can be identified as pathological disease (Javed et al., 2016). High level of NH_3 which can derived from TAN (Table 4) in water may also influence to decrease the red blood cells of Nile tilapia in CF (Table 6) (Ahamed et al., 1992) and the Hct level present in RBCs reduced substantially.

Counting of WBCs is an important indicator of fish health status and immunity towards disease causing agents. It was found that microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. are rich in essential vitamins like, ascorbate (Vitamin C), β-carotene, α-tocopherol (Vitamin E), thiamine, riboflavin, folates (Vitamin B), pyridoxine, cobalamin and biotin (Brown et. al., 1999). However, the presence of retinol (Vitamin A) was only identified in *Tetraselmis* sp. by Brown et. al. (1999). Presence of vitamins in microalgae has acted like immunostimulant in Nile tilapia and it led to the increase of WBCs (Table 6) in cultured fish species to create quick recovery of its body against some stress factors (Amira et.al., 2021). During the culture of Nile tilapia, dietary inclusion of vitamin C and inulin in diet has positively influenced growth performance, inherent immunity, hematology and protection against pathogens in this fish, confirmed by Ibrahem et al. (2010).

The results of LYMs and PLTs were higher in control treatment in the study of Souza et al. (2020). Similarly high level of LYMs and PLTs was recorded in this study (Table 6). It was demonstrated that by reducing the capacity of T-lymphocytes to spread, PUFAs can lower pro-inflammatory reactions. When DHA is administered the number of T lymphocytes suppressor increased then negatively controls other lymphocytes and this is the possible reason of such regulation (Souza et al., 2020). *Tetraselmis* sp. and *Nannochloropsis* sp. microalgae contains high amount of essential n-3 fatty acids, PUFA (Servel et al., 1994). The dietary administration of microalgae containing PUFA is the probable reason of low level of lymphocytes in blood of microalgae fed treatment (Amira et.al., 2021). Platelets in blood is responsible for the creation of protective barrier. However, n-3 PUFA has the quality to cease the platelet quagulation (Thorngren and Gustafson, 1981) and fish get PUFA from microalgae which resulted in low amount of platelet in N50, followed by T50 through this experiment. In this sense, further study needs to clarify this observation (Amira et.al., 2021).

5.5.2 Blood Serum Biochemical Parameters of Nile Tilapia

In this study the results obtained for total serum protein, albumin, globulin, blood glucose and cholesterol are within the reference range (Amira et.al., 2021) provided by Hrubec et al. (2000). However, higher values of albumin, globulin, A/G ratio, cholesterol and urea nitrogen was observed in the study of Mauel et al. (2007), for tilapia fish. During the experiment, Nile tilapia could not successfully derive protein nutrition from feed for both 25% or 50% *Nannochloropsis* sp. and *Tetraselmis* sp. supplementation which was reflected into its blood serum protein, albumin, globulin

and A/G ratio (Figure 6). A lower value of feed protein related parameters was observed in the current study for total protein, albumin and globulin concentration in microalgae fed treatments than control. In certain conditions, the total protein concentration reduces because of reduced synthesis capability, absorption, or protein depletion (Bernet et al., 2001).

In the study of Abd El-Ghany et al. (2020) 5 or 10% supplementation of *Nannochloropsis oculata* for Nile tilapia diet showed higher level of total protein, albumin, globulin and A/G ratio in contrast to control but when supplementation was increased up to 15% of *N. oculata* it resulted in lower values of total protein, albumin, globulin and A/G ratio which is in line with our findings. This study suggests that, microalgae inclusion should be done in lower percentage and not up to 25 or 50% supplementation because it could reduce the protein utilization, absorption capability in fish body resulting in lower serum protein contents in blood.

Although cholesterol and triglyceride are the sources of structural cell membrane component and cellular storage energy for fish health, respectively (Patriche et al., 2011). An increase in these two parameters in blood serum may reflect high storage of lipid and metabolic syndrome (Amira et.al., 2021). In the current study the desired outcome was obtained for cholesterol and triglyceride concentration of reared fish. Microalgae supplementation in this study showed a lowering impact of cholesterol and triglyceride level and it was highly noticed in N50 treatment (Figure 7). Similarly in the study of Al-Deriny et al. (2020) *Spirulina platensis* supplementation in the diet of *Oreochromis niloticus* reduced the cholesterol and triglyceride concentration. Both *Nannochloropsis* sp. and *Tetraselmis suecica* are abundant in n-3 fatty acid EPA, which is 30.1 and 6.2% correspondingly and confirmed by Servel et al. (1994). High EPA in diet could reduce the cholesterol and triglyceride level because EPA like PUFA can lower the concentration of lipid through affecting hyperlipidemia (Sathasivam et al., 2017) and fish got this PUFA from supplemented microalgae in diet in the current study.

Glucose is an instant energy supply that involves heart and muscle activity of the body (Amira et.al., 2021). The quickest and most economical way of assessing the stress situation is to dose the serical glycemia on fish. The cause of significant increase in serum glucose levels in CF (Figure 8) may be environmental stress such as high ammonia, nitrite nitrogen, soluble reactive phosphorus etc. Serum glucose concentration changes are particularly related to renal damage (Coimbra et al., 2000).

Lowest accumulation of stress enzymes can be possible due to addition of microalgal meal in diet (Mukherjee et al., 2019). Moreover, it was found that only 1% *Spirulina platensis* addition in diet of *Oreochromis niloticus* significantly lowered blood glucose level than control treatment in the study of Al-Deriny et al. (2020). Meanwhile, high antioxidant properties present in *Tetraselmis sp.* suppressed the activity of stress producing factors by accelerating quick recovery of its body and thus, lowered the blood glucose concentration in T25 (Figure 8).

Urea is the main food-protein and tissue-protein metabolite. The liver generates nitrogen as the body metabolizes protein (Amira et.al., 2021). To form urea nitrogen which generated from liver binds with other substances derive from liver. After passing through the circulation into the kidney urea drained out of the blood and left in urine (Ajeniyi and Solomon, 2014). Incidentally, BUN reflects only the nitrogen content of urea as waste product. The body removes urea from the urine, which increases the BUN levels as the activity of the kidneys reduces (Amira et.al., 2021). Thus, increase in protein rich diet increases blood urea also (Ajeniyi and Solomon, 2014). In this study, lower feed intake, disturbance in liver protein metabolism in microalgae fed treatment N50, followed by N25 revealed significant ($p < 0.05$) lower urea and BUN concentration in comparison to CF (Figure 9). Urea is mainly released through gills from fish body. Elevation in serum BUN was used to identify fish kidney and gill disorder (Bernet et al., 2001). Elevated level of urea and BUN in CF showing the disturbance of gill function in this regard.

Chapter-6: Conclusions

Nannochloropsis sp. and *Tetraselmis* sp. microalgae species were considered as potential in the aspect of growth, water quality, muscle pigmentation, hemato-biochemical index and nutritional profile. Review of literature in chapter 2 has elaborated the positive characteristics of selected species. Growth, survivality, total carotenoid concentration, hemato-biochemical index, nutritional and biochemical composition of fish mainly depends on the diet provided to fish. Addition of microalgae within fish diets led to an antioxidant enhancement and gives fish a great immunity against bacterial infection and increase the survival rate. This study indicates that, inclusion of *Tetraselmis* sp. and *Nannochloropsis* sp. microalgae in diet created problem regarding palatability resulting in poor growth performance. However, some anti-nutritional factors may also responsible for this. Positive impact on water quality, total carotenoid concentration, hematology was noticed. Influence on some serum parameters was recorded also. Inclusion of *Nannochloropsis* sp. and *Tetraselmis* sp. microalgae in diet has also positively influenced the lipid and carbohydrate deposition, percentages of essential fatty acids in Nile tilapia whole muscle tissue except protein percentage. Lower protein digestibility due to higher inclusion of microalgae, which is a plant protein source may be responsible for this. Considering the beneficial impacts on water quality, survivality, hematology, serum chemistry and nutritional profile, this study provides evidence of two potential microalgae that can be used as a replacement of fish meal for cultivable fish like tilapia. Further research with more treatments and replicates is needed to evaluate more consistently and reliably the negative effect of microalgae on growth and protein percentage of whole tilapia muscle tissue.

Chapter-7: Recommendations and Future perspectives

The current study showed growth, water quality, muscle pigmentation, blood hematology, serum biochemical parameters, proximate and fatty acid composition of fry Nile tilapia (*Oreochromis niloticus*) fed with *Tetraselmis* sp. and *Nannochloropsis* sp. microalgae. The purpose of this study was to identify *Tetraselmis* sp. and *Nannochloropsis* sp. microalgae as a potential source for Nile tilapia fish as feed through determining their beneficial impact on fish health. Although a qualitative approach was followed to explore the objectives of the research, there are some limitations of the study which can be minimized by following recommendations:

- Mass culture of microalgae should be done in a cost-effective way for commercial purpose.
- Biomass of microalgae should be dry in optimal temperature to reduce protein and lipid degradation.
- There should maintain optimum range of physical and chemical parameter for both micro algae and fish culture.
- This study also recommends to lower the inclusion of plant protein source, microalgae to improve the digestibility and protein nutrient deposition.

Future perspectives of this study may include-

- The findings of growth, survival and water quality signifies the idea of microalgae selection as food and for the wellbeing of fish in commercial and scientific purposes. Hemato-biochemical data will aid in diagnosing diseases and examining the degree of blood cell loss to assess health condition and physiological improvements with microalgal diet intake in effective and comprehensive indexes (Amira et.al., 2021).
- The findings will benefit the biologists in understanding the systemic interactions between the homeostasis and physiological modifications consequently from diet and water quality of the fish species so that standard reference values for this fish can be established. Farmers and researchers can utilize the results to improve their yield and to conduct further investigation on the effect of other microalgae species on fish health, disease resistance as well as yield performance, respectively (Amira et.al.,2021).

- Researchers can understand and improve the path of generating alternative way of fish culture for high production and quality improvement in fish farming with commercially important species. Researchers can also improve their research and ensure better success with their culture species. Assessment of the effectiveness of microalgae on the immune system of aquatic organisms can also be designed using these data (Amira et.al., 2021).
- The results of pigmentation will help various food producing company trying to introduce natural pigments of microalgae' in replacement of synthetic colors, on those circumstances the selected microalgae can play a dynamic role. It will also improve the food value of edible fish because pigmentation reduces the dehydration of fish skin and attracts the consumer.
- Impact of lipid and essential fatty acids (like PUFAs) enriched microalgae on fish will help to use microalgae as feed not only for gonadal development of different fishes but also improving the consumer health.

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Appendices

Appendix A: One-way Analysis of Variance examining the growth performance of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

ANOVA Table						
Parameters	**Comparison**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Initial weight (g)	Between Groups	.000	4	.000	1.446	.289
	Within Groups	.000	10	.000		
	Total	.000	14			
Final weight (g)	Between Groups	2.335	4	.584	29354.629	.000
	Within Groups	.000	10	.000		
	Total	2.335	14			
Total weight gain (g)	Between Groups	2.335	4	.584	29277.415	.000
	Within Groups	.000	10	.000		
	Total	2.336	14			
Initial length (cm)	Between Groups	.000	4	.000	.014	1.000.
	Within Groups	.019	10	.002		
	Total	.019	14			
Final length (cm)	Between Groups	1.114	4	.278	480.103	.000
	Within Groups	.006	10	.001		
	Total	1.120	14			
Initial K (g/cm^3)	Between Groups	.019	4	.005	1.446	.289
	Within Groups	.033	10	.003		
	Total	.051	14			
Final K (g/cm^3)	Between Groups	.066	4	.016	16.146	.000
	Within Groups	.010	10	.001		
	Total	.076	14			
SGR (%/day)	Between Groups	3.387	4	.847	766.796	.000
	Within Groups	.011	10	.001		
	Total	3.398	14			
FCR	Between Groups	6.341	4	1.585	17973.480	.000
	Within Groups	.001	10	.000		
	Total	6.342	14			
Survival (%)	Between Groups	1954.733	4	488.683	33.929	.000
	Within Groups	144.033	10	14.403		
	Total	2098.765	14			

Appendix B: One-way Analysis of Variance examining the water quality of treatment tanks of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

Parameters	ANOVA Table					
	Comparison	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
DO (mg/L)	Between Groups	.007	4	.002	.250	.903
	Within Groups	.068	10	.007		
	Total	.075	14			
Temperature (^{0}C)	Between Groups	.093	4	.023	.783	.562
	Within Groups	.297	10	.030		
	Total	.390	14			
pH	Between Groups	.036	4	.009	.776	.566
	Within Groups	.117	10	.012		
	Total	.153	14			
TAN (mg/L)	Between Groups	.064	4	.016	405.415	.000
	Within Groups	.000	10	.000		
	Total	.064	14			
NO_2-N (mg/L)	Between Groups	.017	4	.004	57.802	.000
	Within Groups	.001	10	.000		
	Total	.018	14			
SRP (mg/L)	Between Groups	.005	4	.001	103.665	.000
	Within Groups	.000	10	.000		
	Total	.005	14			

Appendix C: One-way Analysis of Variance examining the muscle pigmentation of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

Concentration	ANOVA Table					
	Comparison	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
380 nm (µg/gram)	Between Groups	2.166	4	.541	126.913	.000
	Within Groups	.043	10	.004		
	Total	2.209	14			
450 nm (µg/gram)	Between Groups	11.696	4	2.924	637.523	.000
	Within Groups	.046	10	.005		
	Total	11.742	14			

470 nm (µg/gram)	Between Groups	14.501	4	3.625	641.255	.000
	Within Groups	.057	10	.006		
	Total	14.557	14			
500 nm (µg/gram)	Between Groups	3.033	4	.758	173.378	.000
	Within Groups	.044	10	.004		
	Total	3.077	14			

Appendix D: One-way Analysis of Variance examining the hematological parameters of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

Parameters	**ANOVA Table**					
	Comparison	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
RBC ($\times 10^6$/µL)	Between Groups	.149	4	.037	66.560	.000
	Within Groups	.006	10	.001		
	Total	.155	14			
Hb (g/dl)	Between Groups	13.871	4	3.468	73.261	.000
	Within Groups	.473	10	.047		
	Total	14.344	14			
Hct (%)	Between Groups	150.433	4	37.608	547.694	.000
	Within Groups	.687	10	.069		
	Total	151.120	14			
WBC ($\times 10^3$/µL)	Between Groups	123.156	4	30.789	164.354	.000
	Within Groups	1.873	10	.187		
	Total	125.029	14			
LYM (%)	Between Groups	293.377	4	73.344	394.324	.000
	Within Groups	1.860	10	.186		
	Total	295.237	14			
PLT ($\times 10^3$/µL)	Between Groups	2333.623	4	583.406	17859.357	.000
	Within Groups	.327	10	.033		
	Total	2333.949	14			

Appendix E: One-way Analysis of Variance examining the blood serum parameters of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

Parameters	ANOVA Table					
	Comparison	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Total Protein (g/dl)	Between Groups	8.436	4	2.109	680.353	.000
	Within Groups	.031	10	.003		
	Total	8.467	14			
Albumin (g/dl)	Between Groups	.557	4	.139	107.046	.000
	Within Groups	.013	10	.001		
	Total	.570	14			
Globulin (g/dl)	Between Groups	4.676	4	1.169	259.373	.000
	Within Groups	.045	10	.005		
	Total	4.721	14			
A/G ratio	Between Groups	.134	4	.033	43.236	.000
	Within Groups	.008	10	.001		
	Total	.141	14			
Triglyceride (mg/dl)	Between Groups	711.589	4	177.897	520.168	.000
	Within Groups	3.420	10	.342		
	Total	715.009	14			
Cholesterol (mg/dl)	Between Groups	4133.506	4	1033.377	4308.009	.000
	Within Groups	2.399	10	.240		
	Total	4135.905	14			
Blood glucose (mg/dl)	Between Groups	282.671	4	70.668	329.197	.000
	Within Groups	2.147	10	.215		
	Total	284.817	14			
Urea (mg/dl)	Between Groups	97.410	4	24.352	72.747	.000
	Within Groups	3.348	10	.335		
	Total	100.757	14			
BUN (mg/dl)	Between Groups	21.244	4	5.311	72.747	.000
	Within Groups	.730	10	.073		
	Total	21.974	14			

Appendix F: One-way Analysis of Variance examining the proximate composition of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

Composition (%)	ANOVA Table					
	Comparison	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Protein	Between Groups	67.331	4	16.833	1759.527	.000
	Within Groups	.096	10	.010		
	Total	67.427	14			
Lipid	Between Groups	93.509	4	23.377	730.542	.000
	Within Groups	.320	10	.032		
	Total	93.829	14			
Carbohydrate	Between Groups	25.224	4	6.306	2840.492	.000
	Within Groups	.022	10	.002		
	Total	25.246	14			

Appendix G: One-way Analysis of Variance examining the fatty acid composition of Nile tilapia *Oreochromis niloticus* after the microalgae used as fish diet

SI. No.	(%) Total fatty acid	ANOVA Table					
		Comparison	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
1.	Octanoic acid	Between Groups	.173	4	.043	18.718	.003
		Within Groups	.012	5	.002		
		Total	.184	9			
2.	Decanoic acid	Between Groups	.190	4	.047	21.196	.002
		Within Groups	.011	5	.002		
		Total	.201	9			
3.	Lauric acid	Between Groups	2.708	4	.677	125.175	.000
		Within Groups	.027	5	.005		
		Total	2.736	9			
4.	Tridecanoic acid	Between Groups	.167	4	.042	91.632	.000
		Within Groups	.002	5	.000		
		Total	.169	9			
5.	Myristic acid	Between Groups	.643	4	.161	56.739	.000

		Within Groups	.014	5	.003		
		Total	.657	9			
6.	Palmitic acid	Between Groups	2.672	4	.668	50.954	.000
		Within Groups	.066	5	.013		
		Total	2.738	9			
7.	Stearic acid	Between Groups	1.268	4	.317	35.829	.001
		Within Groups	.044	5	.009		
		Total	1.312	9			
8.	Arachidic acid	Between Groups	.034	4	.008	9.353	.015
		Within Groups	.005	5	.001		
		Total	.038	9			
9.	Heptadecanoic acid	Between Groups	.046	4	.012	17.856	.004
		Within Groups	.003	5	.001		
		Total	.050	9			
10.	Heneicosanoic acid	Between Groups	.307	4	.077	56.210	.000
		Within Groups	.007	5	.001		
		Total	.314	9			
11.	Behenic acid	Between Groups	.878	4	.219	93.794	.000
		Within Groups	.012	5	.002		
		Total	.889	9			
12.	Tricosanoic acid	Between Groups	.061	4	.015	30.586	.001
		Within Groups	.002	5	.000		
		Total	.063	9			
13.	Lignoceric acid	Between Groups	.682	4	.170	94.182	.000
		Within Groups	.009	5	.002		
		Total	.691	9			
	∑ SFA	Between Groups	10.666	4	2.667	190.984	.000
		Within Groups	.070	5	.014		
		Total	10.736	9			
14.	Palmitoleic acid	Between Groups	1.135	4	.284	44.417	
		Within Groups	.032	5	.006		
		Total	1.167	9			

15.	Oleic acid	Between Groups	15.125	4	3.781	537.391	.000
		Within Groups	.035	5	.007		
		Total	15.160	9			
16.	Euric acid	Between Groups	39.193	4	9.798	2185.531	.000
		Within Groups	.022	5	.004		
		Total	39.215	9			
17.	Cis-11-Eicosenoic acid	Between Groups	.194	4	.048	12.193	.009
		Within Groups	.020	5	.004		
		Total	.214	9			
18.	Nervonic acid	Between Groups	.012	4	.003	10.519	.012
		Within Groups	.001	5	.000		
		Total	.013	9			
	∑ MUFA	Between Groups	14.721	4	3.680	385.236	.000
		Within Groups	.048	5	.010		
		Total	14.769	9			
19.	Linoleic acid	Between Groups	116.811	4	29.203	5388.122	.000
		Within Groups	.027	5	.005		
		Total	116.838	9			
20.	Arachidonic acid	Between Groups	2.588	4	.647	123.016	.000
		Within Groups	.026	5	.005		
		Total	2.614	9			
21.	Eicosatrienoic acid	Between Groups	3.272	4	.818	262.951	.000
		Within Groups	.016	5	.003		
		Total	3.288	9			
22.	Linolenic acid	Between Groups	90.727	4	22.682	1404.399	.000
		Within Groups	.081	5	.016		
		Total	90.807	9			
23.	EPA	Between Groups	100.041	4	25.010	6607.333	.000
		Within Groups	.019	5	.004		
		Total	100.060	9			
24.	DHA	Between Groups	.165	4	.041	604.899	.000

		Within Groups	.000	5	.000		
		Total	.166	9			
25.	DPA	Between Groups	1.159	4	.290	43.624	.000
		Within Groups	.033	5	.007		
		Total	1.193	9			
	∑ PUFA	Between Groups	8.609	4	2.152	67.010	.000
		Within Groups	.161	5	.032		
		Total	8.770	9			
	∑ n-3	Between Groups	45.245	4	11.311	787.658	.000
		Within Groups	.072	5	.014		
		Total	45.316	9			
	∑ n-6	Between Groups	87.297	4	21.824	1171.556	.000
		Within Groups	.093	5	.019		
		Total	87.390	9			
	∑ n-3/n-6	Between Groups	1.024	4	.256	921.822	.000
		Within Groups	.001	5	.000		
		Total	1.026	9			