Chapter-1: Introduction

Ornamental fishes are commercially valued for their external body pigmentation in both national and international fish markets, which determines their price in global trade (Pandey and Mandal, 2017). The development of ornamental fish industry targets getting a higher yield with more vibrant offspring production. Among all the top listed pet fishes, fantail guppy (*Poecilia reticulate*) is the most popular and in rising demand because of its diversified body coloration, unique shade pattern, and fin structure (Sarathchandra et al., 2018). Their ability to adapt to a wide range of environmental and ecological situations has made them popular among hobbyists since they can readily be raised and reproduced in confined spaces (Sarathchandra et al., 2018). In addition, as many fishes cannot be cultured in laboratory settings because of their sensitivity to environmental changes, guppy fish are used as model organisms in a multitude of pilot scale experiments, including ecology, evolution, behavioral studies, and particularly feeding trials (Dernekbasi et al., 2010).

To achieve maximal growth, body coloring, and breeding stimulation, they must obtain a sufficient amount of carotenoids from nutritionally enriched (protein, fat and carbohydrate) supplemented meals (Sinha and Asimi, 2007). Fish meal has been used as the principal source of protein and fat in the manufacturing of fish feed for many years. However, because of its limited supply and higher cost, scientists began to search for an alternative source of nutrition. Microalgae have been considered a successful source of beneficial nutrients and pigmentation in this context, drawing attention to their large-scale production, which began just a few decades ago in the aquaculture sector (Christaki et al., 2011). A small portion of algae in the diet can improve species quality, survival, growth and resistance to disease (Sharma, 2013). Furthermore, there is no evidence that microalgae have a deleterious impact on fish physiology or development (Shi et al., 2013). Plant-based components, on the other hand, are restricted by the existence of anti-nutritional substances that may adversely influence food consumption, growth, fatty acid content, and other physiological activities in fish (Nishshanka et al., 2021).

According to Lupatsch and Gbadamosi (2018), *Nannochloropsis* (Eustigmatophyceae) genera exhibit high protein (43%), lipid (16.6%) and EPA levels (16 percent of total lipid). In addition, eicosapentaenoic acid (EPA) is the most abundant fatty acid (16%)

in *Nannochloropsis*, and it has the potential to partially replace fish meal and oil in commercial fishmeal (Hulatt et al., 2017; Lupatsch and Gbadamosi, 2018). It also has a high photosynthetic efficiency, a digestible simple polysaccharide cell wall, one chloroplast, and is a good source of potential pigments, including chlorophyll, zeaxanthin, canthaxanthin, and astaxanthin (Shah et al., 2018; Lupatsch and Gbadamosi, 2018; Bárbara et al., 2021). In turbot (Qiao et al., 2019) and Atlantic salmon (Sørensen et al., 2017), *Nannochloropsis* sp. has also been linked to reduce lipid peroxidation and increase antioxidant capacity. Furthermore, *Nannochloropsis* sp. could replace up to 10% crude protein from fishmeal and soy concentrate in red drum (*Sciaenops ocellatus*) diets (Patterson and Gatlin, 2013) and 15% fish meal in European seabass diets (Valente et al., 2019) without affecting the fish's growth, feed utilization, protein, or energy retention. As a result, multiple aquaculture industries have shown greater interest in using this microalga to improve the growth performance and feed utilization of many economically valuable aquatic species (Sørensen et al., 2017; Valente et al., 2019; Gong et al., 2020). Different microalgae have been shown to have a favorable influence on guppy growth, survival, coloring, and stress resistance (Dagar et al., 2010; Dernekbasi et al., 2010; Nath et al., 2012; Sarathchandra et al., 2018). However, no research has been carried out on the impact of *Nannochloropsis* on pigmentation, breeding, antioxidants or other biological parameters in this valuable ornamental fish. Therefore, using Guppy (*Poecilia reticulata*) as a model species, the current feeding trial was designed to achieve the following objectives-

i. To evaluate the efficacy of *Nannochloropsis* sp. as a partial replacement of fishmeal on survival and growth performance of guppy
ii. To increase reproductive performance of fish through enhancing body coloration and nutritional enrichment
iii. To determine the efficiency of *Nannochloropsis* in reducing oxidative stress as a potential source of antioxidant

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 natural source of nutrients (protein, lipid, carbohydrate, vitamin etc.) and pigments (chlorophyll, carotenoids, phycobiliproteins etc.) which can be a balanced source of dietary energy for aquatic organisms. A review of literature relevant to the present research work has been given below.

2.1. Significance of Microalgae in Aquaculture

To date, the fastest-growing segment of food industry is aquaculture. In 2019, the aquaculture market is expected to be valued at US$ 31.94 billion (Marketwatch, 2020). Between 2020 and 2027, it is expected to rise at a level of more than 7.1 percent. Aquaculture has expanded the production of fish that depend on plant-based diet. Fish feed has been fine-tuned, leading to fewer wastage and, as a result, it ensures financial sustainability of industry (Kruijssen et al., 2020). The productivity, survival, and quality of cultured fish have all increased thanks to a diet rich in functional elements including antioxidants, omega-3 fatty acids, and prebiotic compounds (Lupatsch and Gbadamosi, 2018). Fish meal is a popular fish feed component because of the following characteristics: (1) excellent solubility and palatability for fish, leading to accelerated development; abnormalities are recorded seldom or not at all; (2) a well-balanced protein, mineral, essential fatty acid, and essential amino acid composition and levels; (3) a low feed conversion ratio (i.e., a significant percentage of feed is transformed into fish biomass), which results in less feed waste; (4) enhanced immunity, resulting in a higher survival rate (Nagappan et al., 2021). In the previous 10 years, demand for fish meal, a vital element in feed, has jumped by 300 percent as the aqua-feed market is growing in tandem with the aquaculture industry. Currently, low-value forage fishes (fish meal) and terrestrial plants provide some of the feed ingredients. The production of fish meal cannot be raised since it would jeopardize the ocean's sustainability and ecosystem. Higher production of terrestrial plant-based feed, on the other hand, results in deforestation and increased freshwater demand. As a response, new and environmentally sustainable feed ingredient sources must be discovered. Thus, various enterprises and scientists are seeking for a sustainable and efficient source to replace

fish meal. Microalgae has been discovered to be a viable alternative supply of balanced nutrients ideal for seedlings and fish development in the expanding aquaculture sector after numerous studies (Hodar et al., 2020).

Microalgae produce more net biomass than any other terrestrial animals or plants (Rizwan et al., 2018). Microalgae, unlike land-based plants, do not require fertile soil to produce; in fact, microalgae may be cultivated in seawater or waste water (Li et al., 2019). In the context of a biorefinery, microalgae might potentially be utilized to produce fish feed (Arun et al., 2020; Nagappan and Nakkeeran, 2020). This notion, for example, might allow for the co-production of important metabolites like pigments with fish diet. The key reason for the microalgae's promise is that it contains the balanced combination of protein, fat, and carbohydrate to safeguard the health of the fish. Microlagae usually have a well-balanced amino acid profile, thus they do not require expensive amino acid supplements in the diet. *Chlorella*, *Porphyridium*, *Chlamydomonas*, *Isochrysis*, and *Nannochloropsis*, for example, are high in methionine, which is likely to be absent in plant-based components (Wan et al., 2019). Microalgal fiber, unlike plant fiber, lacks lignin and has a low hemicellulose content, implying greater digestion (Niccolai et al., 2019). Microalgae also contain a wide range of antioxidant pigments, and certain microalgae create rich vitamins and immunostimulants in their bodies, all of which can benefit aquatic organism’s health (Zhou et al., 2018; Prabha et al., 2020). Astaxanthin, a microalgal pigment, might give fish a more desirable color, enhancing their commercial viability (Posten and Schaub, 2009). Omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosa-pentaenoic acid (EPA) are abundant in some microalgae and are helpful to both fish and humans (Ryckebosch et al., 2012). As a result, selecting microalgal strains with appropriate cellular composition and reduced feed production costs may be able to fulfill a large requirement for fish feed in the near future.

2.2. General Attributes of *Nannochloropsis* sp.

2.2.1. Characteristics and taxonomic classification

Taxonomic classification of *Nannochloropsis* sp.-

Domain : Eukaryote

Kingdom : Viridiplantae

Phylum : Ochrophyta

Class : Eustigmatophyceae

Order : Eustigmatales

Family : Monodopsidaceae

Genus : Nannochloropsis

Nannochloropsis spp. is a microalgae that are related to diatoms and brown algae and living in freshwater and seawater (Andersen et al., 1998; Sukenik et al., 2009) These unicellular algae are 2-5 microns in diameter and spherical or oblong in shape (Kagan and Matulka, 2015). *Nannochloropsis* species are planktonic, unicellular with either 2–4 μm diameter subspherical or 3-4 × 1.5 μm cylindrical cells (Hibbert, 1980) that provide a yellow-green chloroplast with the main pigments being chlorophyll a and the xanthophylls, violaxanthin and vaucheriaxanthin (Lubián et al., 2000). As the genus appears to lack the genes for meiosis thus it never participate in Sexual reproduction (Pan et al., 2011).

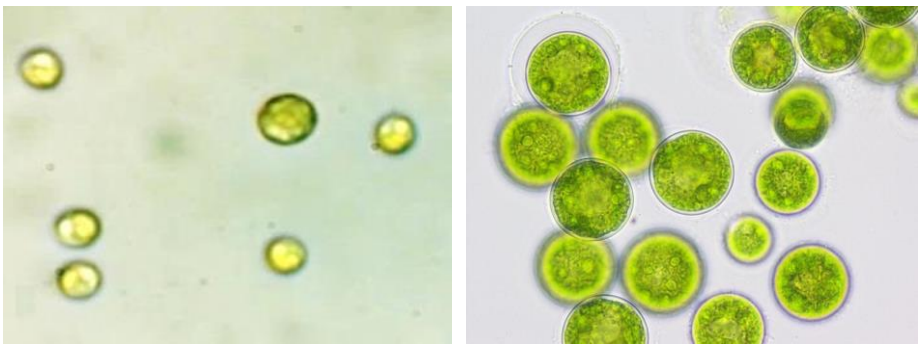


Figure 1.1 Microscopic view of *Nannochloropsis* sp.; a. 40x, b. 100x

2.2.2. Source of nutritional components

Nannochloropsis spp. are the most interesting one among marine microalgae due to their smaller size and high protein content, suitable amino acid profile, and particularly high lipid content (up to 40% of dry matter), typically rich in EPA and DHA (Becker, 2007; Bellou et al., 2014; Molino et al., 2018; Ashour et al,. 2019). Dietary supplementation with 10% *Nannochloropsis* sp. meal exhibited no deleterious impact on fish growth or muscle amino acid composition. In turbot (*Scophthalmus maximus*) (Qiao et al., 2019) or Atlantic salmon (*Salmo salar*) (Sørensen et al., 2017). Moreover, *Nannochloropsis* sp. defatted meal could replace up to 15% fish meal in European seabass (Valente et al., 2019) and up to 10% crude protein from fishmeal and soy concentrate in red drum (*Sciaenops ocellatus*) diets (Patterson and Gatlin, 2013) without affecting growth, feed utilization, protein, and energy retention of the fish.

According to Lupatsch and Gbadamosi (2018), vitamin B12 and Eicosapentaenoic acid (EPA), omega3 HUFAs, protein, carbohydrate, fat, and vitamin C are all found in this species. *Nannochloropsis* (Eustigmatophyceae) taxa have high protein (43%) and lipid (16.6%) content. *Nannochloropsis* spp. have a high methionine content in their protein, which is commonly absent in plant-based components (Wan et al., 2019). Aspartate, glutamate, and proline are the most abundant amino acids, while tryptophan, cystine, histidine, and hydroxyproline are found in small amounts (Scholz et al., 2014). The digestibility of protein available from *Nannochloropsis* sp. in aquatic species ranged from 69 to 81% (Niccolai et al., 2019). Therefore, because of its nutritious composition, the protein-rich biomass of *Nannochloropsis* could be used to substitute fishmeal. The suitability of defatted microalgae as feed ingredients in aquatic animal feeds has been documented by a number of research groups. Patterson and Gatlin (2013) also found that lipid-extracted algal meal (*Nannochloropsis* sp.) could replace up to 10% crude protein from fishmeal and soy protein concentrate in red drum feeds without compromising the fish's growth, feed utilization, protein, and energy retention. *Nannochloropsis* sp. has also been discovered to have the ability to improve Nile tilapia and Grey mullet growth and feed utilization (Jaseera et al., 2021; Abdelghany et al., 2020).

Fatty acids are another characteristic principle of the *Nannochloropsis* sp. biomass; eicosapentaenoic acid (20:5 ω3, EPA) is found in large amounts. Some other fatty acids

also found are 14: 0, 16:0, 16:1, and 20:4 ω6 (Gbadamosi and Lupatsch, 2018). The triacylglycerols are characterized by a high proportion of saturated and monounsaturated short-chain fatty acids 14:0, 14:1, 16: 0, and 16:1. The high quality of *Nannochloropsis* biomass for the aquaculture industry is attributed to its high EPA amount. Among sterol composition, cholesterol, fucosterol, and isofucosterol are the principal constituents in all *Nannochloropsis* species (Creuzburg and Merkel, 2016). In *Nannochloropsis*, eicosapentaenoic acid (EPA) is the abundant fatty acid (16 percent of total lipid) and this characteristic makes the microalga a potential partial fish oil replacer in commercial fishmeal (Hulatt et al., 2017; Lupatsch and Gbadamosi, 2018). It has a high photosynthetic efficiency, a digestible simple polysaccharide cell wall. Also comprises higher fiber content (Niccolai et al., 2019). Glucose is the dominant sugar in the polysaccharide composition (12). *Nannochloropsis* spp. so far not been reported to contain any toxins (Enzing et al., 2014).

2.2.3. Source of valuable pigments

Hibbered was the first to describe the genus *Nannochloropsis* (Kandilian et al., 2013). Members of this genus are characterized by the absence of chlorophyll b, which is a common phenomenon within the class Eustigmatophyceae, and the composition of the cellular xanthophyll pigments (Boussiba et al., 1987; Assaf, 1989). The algae in the genus *Nannochloropsis* vary from other related microalgae in that they only have chlorophyll a and no chlorophyll b or c. *Nannochloropsis* sp. is a significant source of commercially valuable pigments (Macas-Sánchez et al., 2005). *Nannochloropsis* has a simple carotenoid composition, containing the primary xanthophyll pigments carotene, violaxanthin, and a vaucheraxanthin-like pigment (Faé Neto et al., 2018). In cultures exposed to high light intensity, zeaxanthin and anteraxanthin can be found as minor ingredients, both containing ketonic groups (Whittle and Casselton, 1975; Antia et al., 1975; Lubián and Establier, 1982; Karlson et al., 1996). *Nannochloropsis* sp. has a substantially higher concentration of carotenoids than many other microalgae. Also, because the carotenoid molecules found in *Nannochloropsis* spp. are highly assimilable by animals, they are quickly absorbed and transformed into useful colors (Jaseera et al., 2021).

2.2.4. Source of antioxidants

Functional effects of microalgae-based diets on stress response capture a great interest today for both producers and consumers (Elabd et al., 2020; Liu et al., 2020). In a previous investigation, *Nannochloropsis* sp. showed high antioxidant activity (Goh et al., 2010). Because of their capacity to collect high quantities of PUFAs (polyunsaturated fatty acids), they appear to be largely rich in phytochemicals, making them suited for use as a nutritional supplement and natural medicine for the treatment of a variety of illnesses (Matos et al., 2017). The antioxidant enzyme responses in the liver and gut are modulated by *Nannochloropsis* sp. without having a negative impact on total lipid oxidative damage (Castro et al., 2020). *Nannochloropsis* sp. possesses significant polyphenol content associated with non-toxic antioxidant activity, according to Haoujar et al. (2019), which could benefit public health and safety (Day, 2007). Moreover, *Nannochloropsis* sp. is high in carotenoids, which can absorb free radical energy and so help to avoid oxidative damage to tissues (Amaro and Guedes, 2011). In turbot (Qiao et al., 2019) and Atlantic salmon (Sørensen et al., 2017), *Nannochloropsis* sp. has been linked to lower lipid peroxidation and higher antioxidant capacity. A different study found that giving *Nannochloropsis gaditana* to gilthead seabream (*Sparus aurata*) improved their defense activity (Cerezuela et al., 2012).

2.2.5. Growth factors and culture conditions

Nannochloropsis sp. culture is highly dependent upon culture media and environmental factors that influence it (Faria et al., 2012; Boroh et al., 2019). Major factors supporting the microalgal life are light, water, and CO_2 (Elystia et al., 2019). Kitaya et al. (2005) reported the direct effect of several elements, particularly environmental conditions (light, salinity, nutrient types and composition, light period, and culture pattern) on growth and culture of microalgae. According to the review article of Parmar et al. (2011) the length of lighting period and cycle (light/dark) are the most validate factors influenced most in micro-algal cultivation. Most importantly the growth of microalgae affected by some illuminating factors such as length of photoperiod, temperature, pH and light intensity (Wahidin et al., 2013). In order to optimizing microalga growth in mass culture system the above-mentioned factors must have to maintain accurately.

2.2.5.1. Light

Intensity of light is an important factor for microalgae cultivation. Generally, for biomass growth, microalgae depend on enough carbon source (about 40-50% carbon) and light to carry out photosynthesis process (Moheimani, 2005). Requirement varies on basis of the conditions. For an Erlenmeyer flask; 1000 Lux is suitable where 5000-10000 Lux required for larger volume (FAO, 1996). The use of fluorescence light for indoor culture can promote a better growth and cell division of microalgae (Laing, 1991). However, maximum exposure of light can become limiting factor to microalgae density. Kaewpintong (2004) reported cell growth rate increase depending on light intensity, but until a definite stage and after that the growth decrease. It is supported by Lavens and Sorgeloos (1996) that higher light intensity may result in photo-inhibition.

2.2.5.2. Temperature

Temperature, the 2nd most prior factor for culturing microalgae. Besides, above 27 ºC will make the algae die (Laing, 1991). Various microalgae are viable with very low temperature than its optimum (upto 15 ºC lower), but crossing limit above by 1-4 ºC can create a great damage (Teresa et al., 2010). Changing of light intensity will influence temperature which indirectly affects growth of microalgae (Huang et al., 2013). These all factors vary depending upon culture constituents, medium types, species types and strained but temperature ranged between 20-24ºC.

2.2.5.3. pH

pH plays an important role for culturing microalgae. Many cellular activities disrupt in microalgae cell because of pH maintaining failure (Lavens and Sorgeloos, 1996). pH is directly related with CO_2 accessibility and for that reason it is also essential for photosynthesis. In higher concentration pH may varies and reach at limiting values pH 9 (FAO, 1996).

2.2.5.5. Salinity

Salinity has direct effect on growth of microalgae. Salinity range varies on basis of species cultured. Every microalga has a different salinity range. Change in salinity might inhibit the growth of microalgae (Takagi et al., 2006). By adding fresh water or salt we can easily control the salinity of culture medium. Lipids were stored in salt-stressed cells and were degraded when stressed cells are crossed the optimal conditions.

(Shetty et al., 2019). A significant proportional increase in the lipid content of biomass occurred with an increase in salinity (up to 35 ppt), with a concomitant decrease in EPA percentage, in *Nannochloropsis oculata* (Renaud et al., 1999). Complete growth retardation also observed with the higher salinity levels in *Nannochloropsis* sp. (Hu and Gao, 2006); but higher EPA percentage has been noticed with lower brackish water salinities. (Hu and Gao, 2006).

2.2.5.4. Nutrient Composition of Media

Nitrate, phosphate and silicate are some examples of macronutrients essential for microalgae growth (Lavens and Sorgeloos, 1996). Nitrogen is considered as the most important type of nutrient and most common type of nutrient in the culture medium (Thompson et al., 1989). Zinc, cobalt, boron, iron and manganese are the most commonly used trace metals (Probert and Klaas, 1999). Others are thiamin (B1), cyanocobalamin (B12) (FAO, 1996). Day (2006) reports direct interaction of nutrients on growth of microalgae. Commercial media contain all types of micronutrients and macronutrients to help the growth of microalgae.

2.2.5.6. Mixing and Aeration

For homogenizing mixing and aeration is an important factor. Kaewpintong (2004) found fine development of cell growth in an aerated culture system (bioreactor) than non-aerated system. On the contrary, excess liquid pressure, velocity, over turbulence and excess bubbles can create stress to the cell which result damage of cell (Eriksen, 2008). It is also important to prevent sedimentation. So, to ensure better contacts with cell and nutrients it is also important to maintain homogenous conditions through balance aeration.

2.3. Guppy (*Poecilia reticulata*)

The guppy (*Poecilia reticulata*), also known as millionfish or rainbow fish (Fishbase, 2013), is a commonly available tropical fish and among the most popular aquatic aquarium fish species. It's a live-bearing fish that belongs to the Poeciliidae family. Guppies are native to northeastern South America, but they have been adapted to a variety of environments and can now be found worldwide. They are extremely versatile, thriving in a wide range of environmental and biological circumstances (Magurran, 2005). Male guppies have decorative caudal and dorsal fins, which are smaller than

females. In the domains of ecology, evolution, and behavioral sciences, guppies are considered as a model organism (Magurran, 2005).

2.3.1. Taxonomic classification

Guppies were identified as *Poecilia reticulata* by Wilhelm Peters in Venezuela in 1859, while De Filippi named *Lebistes poecilioides* in Barbados in 1861. Albert Günther named it *Girardinus guppii* in memory of Robert John Lechmere Guppy, who brought specimens of the species to the Natural History Museum in London from Trinidad (Günther, 1866). In 1913, Regan reclassified the species *Lebistes reticulatus*. Rosen and Bailey renamed it *Poecilia reticulata* again in 1963. Despite the fact that *Girardinus guppii* is now regarded a minor synonym of *Poecilia reticulata*, "guppy" remains the common name although taxonomy of the species was repeatedly changed (Magurran, 2005).

Taxonomic classification of *Poecilia reticulata* (Guppy)-

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Cyprinodontiformes

Family : Poeciliidae

Genus : Poecilia

Species: *P. reticulata*

2.3.2 Biological features

Sexual dimorphism could be seen in guppies. Males feature streaks, patches, or stripes that can be any of a number of colors, whilst females are grey in appearance (Khoo et al., 1999). Guppies come in a variety of sizes, although males are usually 1.5–3.5 cm (0.6–1.4 in) length, while females are 3–6 cm (1.2–2.4 in). Males feature stunning polymorphic color patterns that include black, yellow, green, white, red-orange, and iridescent dots, bands, and speckles. Males have a gonopdium, which is a narrow, modified anal fin that serves as an intromittent sensor, whereas females have a rounded

anal fin. Females are silver grey in color and have a deeper and wider body than males. Juvenile fish take after females and are self-sufficient from the moment they are born.

Breeders create a range of guppy strains with different shades, patterns, forms, and lengths of fins, like snakeskin and grass types, by selective breeding. Many domestic varieties have morphological characteristics that differ significantly from their wild-type ancestors. Both males and females of many domestic strains have bigger bodies and are more elaborately adorned than their wild-type ancestors (Brooks, 2000).

Figure 2.1 Guppy (*Poecilia reticulata*); a. male, b. female

2.3.3. Habitat and distribution

The small benthopelagic fish *Poecilia reticulata* (Peters, 1860), can be found in a variety of aquatic environments, including lakes, ponds, estuaries, weedy ditches, and canals (Page and Burr, 1991). Guppies are indigenous to Antigua and Barbuda, Suriname, Guyana, Barbados, Trinidad and Tobago, and Venezuela (Fishbase, 2013).

However, guppies have been introduced to many different countries on every continent except Antarctica. Sometimes this has occurred accidentally, but most often for commercial purpose as a popular ornamental fish and a means of mosquito control. They tend to be more abundant in smaller streams and pools than in large, deep, or fast-flowing rivers (Magurran et al., 2001). They are able to tolerate a wide range of aquatic environments and conditions. Guppies have tolerance to brackish water and have colonized some brackish environments (Magurran, 2005). They also are capable of being acclimated to full saltwater as well as being used to cycle saltwater aquariums like their molly cousins.

2.3.4. Food and feeding habit

The guppy is an omnivore fish that consumes both vegetable and animal matter in its diet (Skelton, 1993). Algal remnants, diatoms, crustaceans, plant debris, aquatic insect larvae, mineral particles, as well as other forms of food are consumed by wild guppies. In most occasions, algal remnants make up the majority of a wild guppy's diet, however diets vary based on the specific requirements of food availability in the ecosystem. (Dussault et al., 1981; Lawal et al., 2012). Guppy's foraging and feeding behavior are influenced by the size of the group (Schmidt, 2002) and the participation of individuals of the opposing sex (Griffiths, 1996). The most essential signals that impact on their eating are chemical stimulation from the diet, physical texture, and particle size (Noakes and Baylis, 1990). The food that the fish consumes is also influenced by the gape size of guppy. According to many research, different types of guppies exhibit weak and fluctuating feeding preferences on variable environmental conditions.

2.3.5. Reproductive biology

Guppies are highly prolific livebearers (Fishbase, 2013). The gestation period of guppies varies considerably, ranging from 20 to 60 days at 25 to 27 °C and depending on several environmental factors (Evans and Magurran, 2000; Sato et al., 2021). Reproduction typically continues through the year, and the female becomes ready for conception again quickly after parturition (Magurran, 2005).

In courted mating, where the female shows receptive behavior following the male's courtship display, the male briefly inserts the gonopodium into the female's genital pore for internal fertilization. Once inseminated, female guppies can store sperm in their ovaries and gonoducts, which can continue to fertilize ova up to eight months (Winge, 1937). Because of the sperm-storage mechanism, males are capable of posthumous reproduction, meaning the female mate can give birth to the male's offspring long after the male's death, which contributes significantly to the reproductive dynamics of the wild guppy populations (López-Sepulcre et al., 2013). Pregnant female guppies have enlarged and darkened gravid spots near their anal vents. Just before birth, the eyes of fry may be seen through the translucent skin in this area of the female's body (Mayntz, 2013). When birth occurs, individual offspring are dropped in sequence, typically over a period of one to six hours. The female guppy has drops of two to 200 fry at a time, typically ranging between 30 and 60 (Donovan, 2013).

2.3.6. Rearing conditions in aquarium

Guppies prefer a hard-water aquarium with a temperature between 25.5 and 27.8 °C (78 and 82 °F) and salt levels equivalent to one tablespoon per 19 L (Hargrove and Hargrove, 2006). They can withstand levels of salinity up to 150% that of normal seawater (Corraze, 1993), which has led to them being occasionally included in marine tropical community tanks, as well as in freshwater tropical tanks. Its most famous characteristic is its propensity for breeding, and it can breed in both freshwater and marine aquaria (Takahito and Yoshihisa, 1997). They cannot tolerate water temperatures below 15°C, and is only found in temperate regions in artificially warmed water bodies, for example at cooling lakes of power stations. Critical thermal maxima ranging of 39-41°C and death points of 41-43°C were reported by Chung (2004).

Well-fed adults do not often eat their own young, although sometimes safe zones are required for the fry. Specially designed livebearer birthing tanks, which can be suspended inside the aquarium, are available from aquatic retailers. These also serve to shield the pregnant female from further attention from the males, which is important because the males sometimes attack the females while they are giving birth (Donovan, 2013). It also provides a separate area for the newborn young as protection from being eaten by their mother.

2.3.7. Global trade and economic value of guppy

The value of international trade in ornamental fish in 1992 was US$247 million, based on import statistics, or US$140 million, based on export statistics. More than 100 countries are involved in this trade. Over 2500 species of fish are involved, of which over 60% are of freshwater origin (Globe Newswire, 2021). *P. reticulata* is one of the most popular aquarium fishes, and has been in the ornamental fish hobby since the early 1900s. To date, the most popular species remains guppy, constituting more than 28% of the market. *P. reticulata* has considerable economic value as an ornamental aquarium species, and is widely cultured in commercial fish hatcheries. It has high recreational and aesthetic value in captivity, but has little or no social benefit in its feral state and is not suitable for recreational fishing.

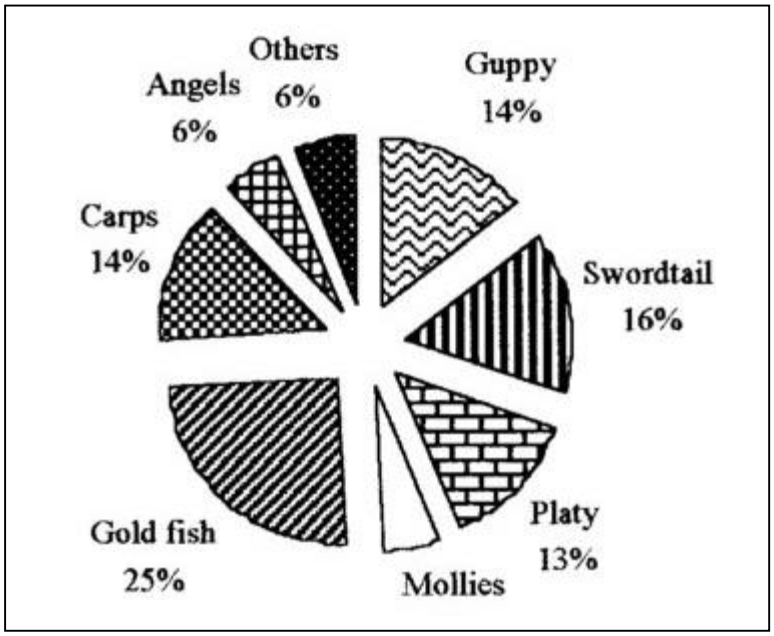


Figure 3.1 International market demand for ornamental fish

Around the world, a number of highly ornamented aquarium strains have been developed, and are extremely popular in the retail aquarium trade, being carried by up to 95% of pet shops. Major producers include Singapore, Malaysia and Taiwan. While initially thought to be a good candidate for mosquito control, *P. reticulata* has had mixed success in controlling mosquito populations (Juliano et al., 1989; Castleberry and Cech, 1990).

Chapter-3: Materials and Methods

3.1. Culture of algal biomass (*Nannochloropsis* sp.)

3.1.1. Collection and preparation of pure inoculums

Pure isolates of *Nannochloropsis* sp., were obtained from laboratory of live feed research corner, Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. The microalgae isolates were inoculated in Conway medium (Table 1) using the formula described by Tompkins et al. (1995) where 100 ml mother culture was inoculated in 900 ml of Conway medium to maintain the stock solution. Then cultures were kept in a thermo-statically controlled room and incubated for 15 days at 24°C with cool inflorescence lamps (Phillips 40 W, cool daylight 6500 K) at an intensity of 2000 lux in a 12: 12 h light dark regime (Bleakley and Hayes, 2017).

Table 1 Chemical composition of Conway medium

Solution A-Macronutrients	
Compound name and molecular formula	Proportions
Sodium/Potassium nitrate ($NaNO_3$ /KNO_3)	10 0.0 0 g/116.0 0 g
EDTA Disodium salt ($C_{10}H_{16}N_2O_8$)	45.00 g
Boric acid (H_3BO_3)	33.60 g
Sodium di-hydrogen orthophosphate ($NaH_2PO_4.4H_2O$)	20.00 g
Ferric chloride hexahydrate ($FeCL_3.6H_2O$)	1.30 g
Manganese (II) chloride tetrahydrate ($MnCL_2.4H_2O$)	0.36 g
Deionized/distilled water	1 L
Solution B-Trace metal solution	
Compound name and molecular formula	Proportions
Zinc chloride ($ZnCl_2$)	2.10 g
Cobalt (II) chloride hexahydrate ($CoCl_3.6H_2O$)	2.00 g
Ammonium molybdate tetrahydrate ($(NH_4)_6MO_7O_{24}.4H_2O$)	0.90 g
Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$)	2.00 g
Zinc chloride ($ZnCl_2$)	2.10 g
Deionized/distilled water	1 L

Solution C-Vitamin's solution	
Compound name and molecular formula	Proportions
Thiamine, Vitamin B1	200 mg
Cyanocobalamin, Vitamin B12	10 mg
Deionized/distilled water	100 mL

3.1.2 Mass Culture in glass tanks

Sea water was collected from Cox's Bazar to prepare media for mass culture. To eliminate debris, the collected sea water was first stored in plastic tanks overnight to allow the solid particles to settle down appropriately; filtered with a filter bag and vacuum filter pump to separate fine particles from the water and then sterilized in an autoclave (for 15 mins in 121℃ and 15 lbs). Then, the growth media was prepared with required nutrients; salinity and pH were adjusted to 26 ppt and 7.8, optimum for the culture of *Nannochloropsis* sp. (Islam et al., 2021). Pure strains from the stock were transferred to 20L glass tanks for mass culture at room temperature with a 12 hour light: 12 hour dark photoperiod, commencing with a volume of 5L and subsequently scaling up to 16 L. Every two days, media was added to the culture tank until it reached the desired volume. Individual culture tanks was supplied with continuous aeration through a central air pump. Transparent polythene was used to cover the top sides of culture tanks to prevent contamination. Every week, samples from each tank were taken and examined under a light microscope for any signs of contamination. Temperature within the algal culture unit was measured daily.

3.1.3. Harvesting as dry algal biomass

A 16-day culture trial was used to determine the native strain's stationary phase in a previous investigation (Islam et al., 2021). As a result, algal biomass was recovered by centrifugation (Muylaert et al., 2017) during the previously reported stationary phase and dried in a hot air oven (Natural Convention Oven LNO-150) at 60°C overnight (Stramarkou et al., 2017). The dried biomass was pulverized using small sized mortar and pestle. The finely powdered algal biomass was then stored in airtight glass vials at 4°C temperature until it could be used in the production of test diets.

3.2. Experimental design

3.2.1. Test diet preparation

All of the formulated test diets were approximately iso-proteic and iso-lipidic (Crude protein-41% and lipid-8 %). The necessary feed ingredients such as, processed feed grade fishmeal, rice bran, wheat flour, vitamin+mineral premix, and commercial feed was purchased from local stores in Chattogram City (Bangladesh). Proximate composition of *Nannochloropsis* sp. was determined before experimental diet formulation (Table 2). First, fishmeal and rice bran were sieved and oven-dried at 60ºC to reduce the moisture level and crushed to make more fine powder. To make dough, fishmeal, rice bran and wheat flour were mixed together and then *Nannochloropsis* sp. powder was added to that mixture as required. For comparative study, dried *Nannochloropsis* sp. powder was incorporated into the diet by replacing fishmeal and other ingredients as follows: control (no inclusion), 5% (N5), 10% (N10), and 15% (N15) for comparative study. The composition of the base ration used in the diet is shown in Table 3. After mixing all of the ingredients together, an adequate amount of water was added to form a doughy texture. Then the doughy mixture was run through a grinder, dried, and granulated. The diets were sieved to ensure uniform granule size. Before final approval, all of the experimental diets were subjected to a chemical analysis. Crude protein (Kjeldahl Auto System) (ISO 5983–1987) and crude lipid (Soxtec HT6) of all the formulated feeds were determined to ensure homogenous nutritional quality (Table 3). Finally, the prepared feeds were sealed in plastic bags, placed in airtight, labeled containers, and stored in a cool (4°C), dry place away from direct sunlight to preserve the feed's quality.

Table 2 Biochemical properties of *Nannochloropsis* sp.

Parameter	Concentration
Crude protein	49 ± 2.28% dry weight
Crude lipid	25 ± 1.84% dry weight
Carbohydrate	25 ± 1.84% dry weight
Chlorophyll	0.536 ± 0.048 µg/L
Carotenoid	1.68 ± 0.3 µg/mL

Table 3 Percent ingredients and proximate composition of the experimental diets.

	Control	Microalgae diet			Commercial
		N5	N10	N15	CMF
Ingredients					
Fish meal	62%	60%	58%	56%	-
Rice bran	28%	25%	22%	19%	-
Wheat flour	8%	8%	8%	8%	-
vitamins + minerals	2%	2%	2%	2%	-
Microalgae powder (*Nannochloropsis* sp.)	-	5%	10%	15%	-
Proximate composition					
Crude protein (%)	41.32%	41.29%	41.26%	41.23%	41.32%
Lipid (%)	8.14%	8.21%	8.23%	8.28%	7.9%

3.2.2. Collection and stocking of fish

Guppy fries (*Poecilia reticulate*) were purchased from a local aquarium shop and brought to the lab in aerated plastic bags. Then the plastic bags were kept in the previously prepared (filtered, aerated and UV treated for 2 days) conditioning tank for three hours. Afterwards, the fries were gently released from the bag in the tank water and kept there for three days to being acclimatized with the laboratory conditions. Potassium permanganate ($KMnO_4$) was used to disinfect the newly brought fries. This will reduce the mortality rate during experimental period. Fries with an average weight of 70 ± 15 mg were selected for the study. Then the fries were divided into five treatment groups, each with a triplicate replication cohort of fifteen uniformly sized individuals. Then each group were kept in a rectangular glass tank ($18\times 12\times 14$ inches) filled with 20L of water maintaining the male and female ratio to 1:2 in an indoor setting.

3.2.3. Feeding experiment

Test diets were fed to the fish at 5% of their body weight twice a day in equally divided doses at 6 hour intervals (10:00 AM and 4 PM). Throughout the trial, each tank received continuous aeration. To maintain optimum water quality, every day one third portion of the culture water was siphoned out from the tank bottom followed by adding new water to compensate the removed amount. A complete water exchange in the tanks was done twice per week.

Water temperature was maintained at 26 ± 2℃ with a 12 h dark: 12 h light photoperiod, dissolved oxygen at 6.5±1 mg and pH at 7.7 to 8.4. Required amount of lime was used to maintain the pH of the culture tank whenever needed. Total ammonia and nitrite concentrations remained below 0.02 mg. This study was carried out over 100 days to cover the growth and reproduction stages (21-day old to 120-day old) of guppy fish.

3.2.4. Daily monitoring and record keeping

On a daily basis, water quality parameters such as temperature, pH and dissolved oxygen (DO; mg/L) was measured with glass thermometer, a handheld pH meter (pHep-HI98107, HANNA, Romania), and dissolved oxygen meter (DO-5509, Lutron). Mortality rate and breeding status were observed on a daily basis. Fish were also routinely monitored for any occurrence of stress or disease outbreak. Dead fish were removed from the aquarium as soon as they were discovered.

3.3. Data collection and analyses

3.3.1. Assessment of growth parameters

At the termination of feeding trial, fish were starved for 24 hours before sampling to empty their gastrointestinal system and then anesthetized with clove oil (eugenol solution) to reduce the handling stress while sampling. Fish were randomly selected from each tank and weighed using a digital meter for growth study. The metrics utilized to evaluate fish growth performance included net weight gain, average daily gain, specific growth rate, length increment, condition factor, FCR, and FCE. Growth parameters were calculated using the following formula (Sarathchandra et al., 2018; Ayala et al., 2020)-

Survival rate (%) = (Number of harvested fish/ Number of fish stocked) × 100

ADG (Average Daily Gain) = (Final weight − Initial weight) / culture period

SGR (%) = ln (final weight) − ln (initial weight) / Duration of experiment (days) ×100

Length increment (cm) = Final total length (cm) - Primary total length (cm)

Condition factor (g cm^{-3}) = [Body weight (g) / Body $length^3$ (cm)] × 100

FCR (g⁄g) = feed given (dry weight) / Body weight gain (wet weight)

3.3.2. Proximate composition analysis

Sample fish were dried in a hot air oven for 8 hours at 40°C to determine carcass proximate composition. The dried carcass was then finely ground and homogenized with a mortar and pestle prior to the analyses of crude protein, crude lipid and carbohydrate content.

3.3.2.1 Protein

Following the method of Lowry et al. (1951), the protein analysis was performed. First, reactive 1 (1 percent potassium sodium tartrate) and Reactive 2 (2 g sodium carbonate per 100 ml 0.1 N NaOH) were prepared. Then, 1 mL of Reactive 1 was added to 50 mL of Reactive 2 to prepare a final volume of mixed reagent. For each sample analysis, 5 to 6 mg of oven-dried sample was taken, and a 25 ml solution was made by combining it with deionized water. 0.5 ml of aliquot from each sample was collected from the generated 25 ml sample. 0.5 ml of sample was mixed with 0.5 ml of 1 N sodium hydroxide and maintained in a water bath at 100 °C for 5 minutes. It was then chilled in a cold water bath for 10 minutes before adding the prepared mixed reagent. Afterwards, 2.5 ml of the mixed solution was mixed with 0.5 mL Folin reagent and stored in the dark for 30 minutes. In a spectrophotometer (T80 UV/VIS Spectrophotometer), absorbance of the mixed reagent was measured at 750 nm wavelength.

3.3.2.2. Lipid

Lipid content was determined according to Bligh and Dyer (1959) and Folch et al. (1957). For each sample, aluminum dishes were constructed and labeled. Each labeled dish's initial weight was recorded. A pre-weighed 50 mg sample was taken in a centrifuge tube and diluted 5 times in deionized water. The sample was systematically mixed with 3 ml 1:2 chloroform: methanol (v/v) using a tissue homogenizer. Then the

mixed solution was centrifuged at 1000 rpm for 4 minutes at 4 ℃ temperature. Supernatants were transferred to a clean centrifuge tube and stored on ice using a Pasteur pipette. 3 ml of 2:1 methanol: chloroform (v/v) was consistently mixed with the sample. The tubes were centrifuged for one more time, and the supernatants were transferred to the old supernatant tubes. In this combined supernatant, 1.5 ml of 0.9 percent NaCl was mixed with a vortex mixture (VM-10). After that, the tubes were placed in the refrigerated for an hour at 4 °C. After an hour, the tubes were centrifuged at 4 °C for 10 minutes at 1000 rpm .Two distinct layer of solution were formed inside the centrifuge tube. First, the upper layer containing methanol and chloroform was removed with a Pasteur pipette and then the lower layer was transferred to the aluminium dish. The solvent in the dishes was evaporated in a hot air oven at 4°C temperature. The final weight was determined by weighing the aluminum plate. Finally, the lipid weight of the samples was calculated by subtracting the initial weight from the final weight.

3.3.2.3. Carbohydrate

Carbohydrate analysis was carried out using the method of Dubios et al. (1956). 5-6 mg of oven-dried microalgae sample was taken for each assay, and a 25 ml solution was made by mixing it with deionized water. Prior to the analysis, a 5 percent phenol solution and concentrated sulphuric acid were prepared. From the prepared sample, a milliliter aliquot was taken into the test tube. The test tube was filled with one milliliter of phenolic solution and five milliliters of strong sulphuric acid and placed in an ice bath to cool. After cooling, the optical density was measured with a spectrophotometer (T80 UV/VIS Spectrophotometer) at 488 nm wavelength.

3.3.3. Determination of total carotenoid

The pigment extraction process described by Olson (1979) was used to extract fin and muscle from the beheaded and degutted fish body immediately after the experiment was completed. One gram of sample from each treatment group was mixed with 2.5 g of anhydrous sodium sulfate in a 10 ml screw-capped clear glass vial and gently meshed with a glass rod. After adding 5 mL chloroform to each vial, they were kept in the refrigerator at 0℃ temperature for overnight. When two distinct layers of chloroform and cake residue had formed, 0.3 mL clear aliquots of chloroform were diluted to 3 mL with 100% ethanol. In a spectrophotometer, the optical density of the prepared solution

was measured at 380, 450, 470, and 500 nm, but only the wave length with the maximum absorbance value was recorded and used in the calculations.

Mukherjee et al. (2009) calculated the total carotenoid content as g per weight of tissue as follows:

$$\text{Total carotenoid concentration (µg/g wet wt.)} = \frac{\text{Absorption at maximum wave length}}{0.25 \times \text{sample weight (g)} \times 10}$$

Where, 10 = dilution factor; 0.25 = Extinction coefficient.

$$\text{Total carotenoid concentration (\%)} = \frac{\text{Absorption at maximum wave length}}{0.25 \times \text{sample weight (g)} \times 10} \times 100$$

$$= Y$$

Generally, Astaxanthin can range from 80 to 100 percent of the total carotenoids.

$$\text{Astaxanthin (\%)} = \frac{Y}{80} \times 100$$

3.3.4. Analysis of oxidative stress biomarker

3.3.4.1. Hydrogen peroxide assay

The level of hydrogen peroxide (H_2O_2) in fish tissue was tested on the last day of culture (Velikova et al., 2000). Fresh fish tissue (0.15 g) was homogenized with 1.0 mL of 0.1% trichloroacetic acid (TCA) before being centrifuged for 10 minutes at 10,000 rpm. Then, 0.5 mL of supernatant was mixed with 0.5 mL of potassium phosphate buffer (10 mM, pH 7.0). After adding 1 mL potassium iodide to the mixture, the solution was incubated under dim light for 10 minutes. The absorbance was measured at 390 nm using a spectrophotometer.

3.3.4.2. Lipid peroxidation assay

The level of lipid peroxidation was evaluated following Heath and Packer's method (1968). Fresh fish (0.15 g) were mixed with 1.0 mL of 0.1% TCA solution and homogenized before centrifuging at 10,000 rpm for 5 minutes. 2.25 mL of the TBA reagent was then added to 0.75 mL of the supernatant. 0.5 g TBA was dissolved in 100 mL of 20% TCA to make the TBA reagent. Then the mixture was placed in a hot water

bath for 30 minutes at 95°C temperature and immediately chilled in an ice bath for 15 minutes. After centrifugation at 10,000 rpm for 10 minutes, the absorbance was measured at 532 nm and 600 nm in the spectrophotometer.

Lipid peroxidation level was determined as equivalent to Malondialdehyde (MDA) and calculated as follows:

$$\text{MDA equivalent (nmol g}^{-1}\text{ FW)} = \frac{A532 - A600}{15{,}5000} \times 10^6$$

Where, A532 = absorbance at 532 nm

A600 = absorbance at 600 nm.

The results were expressed as nanomole MDA/g fresh weight of tissue sample.

3.3.5. Breeding performance

During the experimental period fish were monitored on a daily basis to identify and isolate the gravid female guppy in a separate breeding tank. There, the gravid fish were kept in a submerged net like structure so that when the fry comes out it can escape through the mesh which will reduce cannibalism resulting in high survivability of offspring. After they gave birth, the fish were returned to their corresponding tanks for further mating and the fries born were collected from the breeding tank and transferred to separate nursery tanks.

3.3.6. Chemical analysis of water quality

3.3.6.1. Total ammonium nitrogen (TAN)

Total ammonia nitrogen was determined according to Parson et al. (1984). Standard stock solution was prepared by weighing 9.343 g of anhydrous grade $(NH_4)_2SO_4$ (dried at 110℃ for 1 hour, cooled in dessicator before weighing) and dissolving in 1000 ml deionized water. From the stock solution (1000 mg L^{-1} of total ammonia-nitrogen), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) were prepared by mixing with appropriate ratio of deionized water. Samples and standard solutions (10 ml) were placed in test tube and 0.4 ml of phenol solution (20 g of analytical grade phenol was dissolved in 200 ml of 95% v/v ethyl alcohol and 0.4 ml of sodium nitroprusside (1 g of $Na_2[Fe(CN)_5\ NO]_2H_2O$, dissolved in 200 ml of DDH_2O water) was added in sequence.

Finally, 1 ml of oxidizing solution was added and allows cooling at room temperature (20-27℃) for 1 hour. The test tubes were covered with parafilm (the color is stable for 24 hours after the reaction period). The extinction was measured at 640 nm with Shimadzu spectrophotometer model UV-1601. Oxidizing solution was prepared by mixing 100 ml of alkaline reagent (dissolve 100 g of sodium citrate and 5 g of sodium hydroxide in 500 ml of DDH_2O) and 25 ml of sodium hypochlorite solution [commercial hypochlorite (e.g. clorox) which should be about 1.5 N].

3.3.6.2. Nitrite-nitrogen (NO_2-N)

Nitrite was determined according to Parsons et al. (1984). Standard stock solution was prepared by weighing 4.9259 g anhydrous grade $NaNO_2$ (dried at 110℃ for 1 hour, cooled in dessicator before weighing) and dissolving in 1000 mL deionized water. From the stock solution (1000 mg L^{-1} of NO_2-N), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) were prepared by mixing with deionized water.

Samples and standard solutions (10 ml) were placed in test tube. Then 0.2 ml of sulfanilamide solution (5 g of sulfanilamide was dissolved in a mixture of 50 mL of concentrated hydrochloric acid and dilute to 500 ml with DDH_2O) was added. After more than 2 minutes but less than 10 minutes, 1 ml of NED reagent (0.5 g of the N- (1-napthyl)-ethylenediamine dihydrochloride was dissolved in 500 ml of distilled water) was added and mixed immediately. Between 10 minutes and 2 hours afterwards, the extinction was measured at a wavelength of 543 nm by using the spectrophotometer (T80 UV/VIS Spectrophotometer).

3.3.6.3. Soluble reactive phosphorous (SRP)

Soluble reactive phosphorous (SRP) was determined according to Parsons et al. (1984). Standard stock solution was prepared by weighing 4.3937 g of anhydrous grade potassium dihydrogen phosphate, KH_2PO_4 (dried at 110℃ for 1 hour, cooled in dessicator before weighing) and dissolving in 1000 ml deionized water. From the stock solution (1000 mg $L^{-1}$of PO_4-P) a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) was prepared by mixing with deionized water.

Ten milliliter of samples and standard solutions (10 ml) were placed in test tubes and 1 ml of mixed reagent was added. After 5 minutes and preferably within the first 2-3 hours, the extinction was measured at 885 nm by using Shimadzu spectrophotometer

model UV-1601. Mixed reagent was prepared by mixing 100 ml of ammonium molybdate (dissolve 15 g of analytical reagent grade ammonium paramolybdate $(NH_4)6Mo7O24. 4H_2O$ in 500 ml of distilled water), 250 ml sulfuric acid, 100 ml ascorbic acid (dissolve 27 g of ascorbic acid in 500 ml of distilled water) and 50 ml of potassium antimonyl-tartrate solution (dissolve 0.34 g of potassium antimonyl-tartrate (tartar emetic) in 250 ml of water.

3.3.7. Statistical analysis

The mean and standard error of the mean of all the data were calculated in MS Excel and reported throughout the text as means and standard error. The IBM SPSS (v. 26.0) software was used to perform all statistical analyses related to the survival rate, growth characteristics, proximate composition, pigments, and oxidative stress. Descriptive statistics were computed for each treatment, followed by a test for homogeneity of variance. A one-way analysis of variance was used to examine the acquired data (ANOVA). Percentage data was transformed before performing ANOVA. Tukey's multiple comparison tests were used to look for significant differences among treatments at 95% confidence interval level. To distinguish between groups, a post-hoc test was performed.

Chapter-4: Results

4.1. Growth performance

At the end of feeding trial, the guppy appeared healthy, with no significant ($P > 0.05$) differences in SGR or FCR across all diets. Total number of live fish from each treatment tanks were recorded. The results showed that survival rate was higher in the groups fed with *Nannochloropsis* sp. incorporated diets. Highest survival rate was found in N15, then N10 and N5 respectively (86.7%, 80% and 68.9%) compared with the control group (66.75%). The survival rate was found to be very low (51.1%) in the group fed with commercial diet (CMF) than all other groups.

Average daily gain was in the range of 0.028 – 0.034 mg and SGR ranged from 1.035 to 1.219%. Feed conversion ratios (FCR) were within the range of 3.502 – 3.699. Length increment was within the range of 1.79 - 1.98 cm and condition factor ranged 0.799 to 0.885 g cm^{-3} among all dietary treatments. There were no significant ($P > 0.05$) differences found among control, N5 and N10 in terms of ADG and SGR, whereas N15 and CMF were found to be significantly lower (Table 4). Length increment, condition factor and FCR values were almost similar among treatment groups except for CMF, which showed significantly lower growth performance.

Table 4 Different growth indices of guppy fed with experimental diets.

Parameters	Control	N5	N10	N15	CMF
Survival rate (%)	66.67 ± 4.71	68.89 ± 2.72	80.00 ± 4.71	86.67 ± 4.71	51.11 ± 2.72
Initial length (cm)	0.83 ± 0.04	0.87 ± 0.04	0.83 ± 0.04	0.83 ± 0.04	0.80 ± 00
Final length (cm)	2.79 ± 0.01	2.81 ± 0.01	2.82 ± 00	2.79 ± 00	2.60 ± 0.04
LI[1] (cm)	1.957 ± 0.02[a]	1.944 ± 0.03[a]	1.989 ± 0.03[a]	1.957 ± 0.03[a]	1.798± 0.02[b]
Initial weight (mg)	55.90 ± 0.62	58.20 ± 0.2	58.28 ± 0.78	60.09 ± 0.14	49.67 ± 0.53

Final weight (mg)	189.10 ± 0.63	196.59 ± 0.65	196.21 ± 0.57	187.28 ± 0.39	139.87 ± 0.78
ADG[2] (mg d^{-1})	0.034 ± 0.00[a]	0.034 ± 0.00[a]	0.034 ± 0.00[a]	0.031 ± 0.00[b]	0.028 ± 0.00[c]
Condition factor (g cm^{-3})	0.870 ± 0.003[a]	0.885 ± 0.001[a]	0.873 ± 0.002[a]	0.862 ± 0.009[a]	0.799 ± 0.01[b]
SGR[3] (% d^{-1})	1.219 ± 0.007[a]	1.217 ± 0.005[a]	1.214 ± 0.008[a]	1.137 ± 0.003[b]	1.035 ± 0.01[c]
FCR[4]	3.699 ± 0.05[a]	3.554 ± 0.003[ab]	3.502 ± 0.04[ab]	3.533 ± 0.003[ab]	3.564 ± 0.04[b]

Note: Values are means of ± SE of three replicate groups (n = 3); [1]LI, Length increment; [2]ADG, Average daily gain; [3]SGR, Specific growth rate; [4]FCR, Feed conversion ratio

4.3. Carcass proximate composition

The data illustration in Figure 4.3 showed that N15 group has the highest lipid value (23.66%) and CMF group has the lowest value (16.67%). But the result was almost similar in the cases of N15 and N10. No significant difference ($P > 0.05$) has been found between N5 and control groups. Moreover, there wasn't a distinct statistical difference in protein retention between the control and treatment groups ($P > 0.05$), except that the commercial feed fed fishes showed a significantly lower amount of protein content (34.62%). The results also indicate that the value of carbohydrate was significantly lower (12.89%) in control one than in the other groups ($P < 0.05$), whereas there were no statistical differences found among the treatment and commercial feed fed groups ($P > 0.05$).

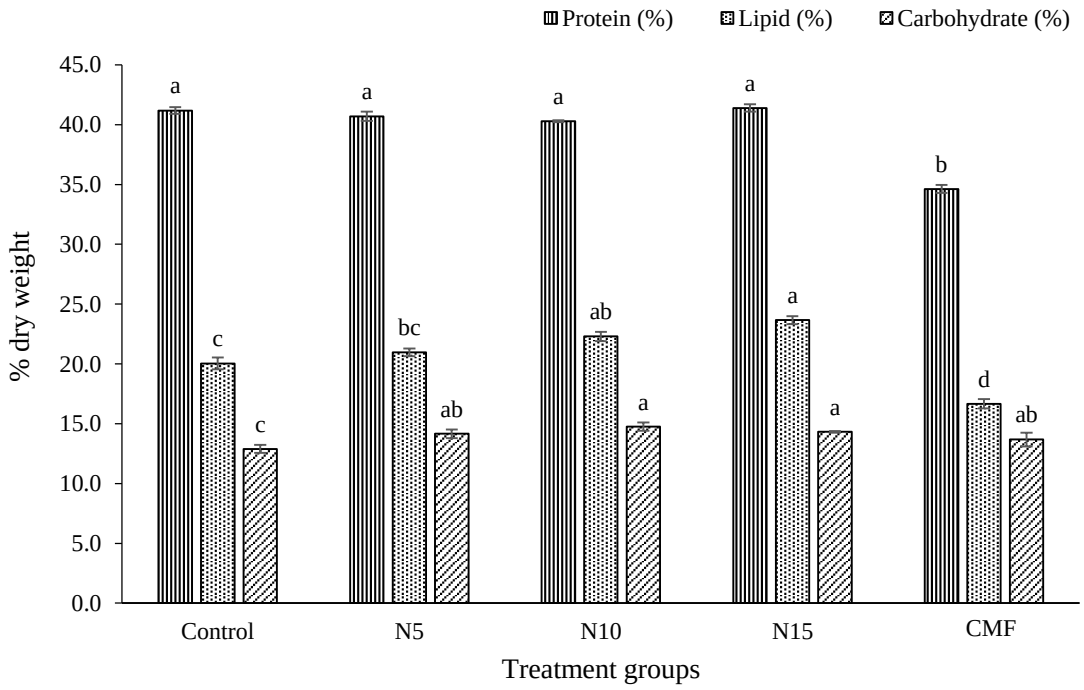


Figure 4.3 Proximate composition (% dry weight) of whole fish body fed with different diets at the end of experiment.

4.4. Total carotenoid and astaxanthin

At the time of termination, total carotenoid and astaxanthin content of the experimental fish showed significant variation (Figure 4.4) among treatment groups ($P < 0.05$). The best coloration was observed in fin and muscle content of the fishes fed with 15% *Nannochloropsis* sp. inclusion feed, followed by 10% and 5% microalgae containing feed fed groups (3.68 μg g^{-1}, 2.56 μg g-1 and 2.88 μg g^{-1}). The total carotenoid accumulation was detected to be significantly lower in the control group than in the treatment groups (2.16 μg g^{-1}). The lowest pigment concentration was found in the commercial feed fed group (1.67 μg g^{-1}).

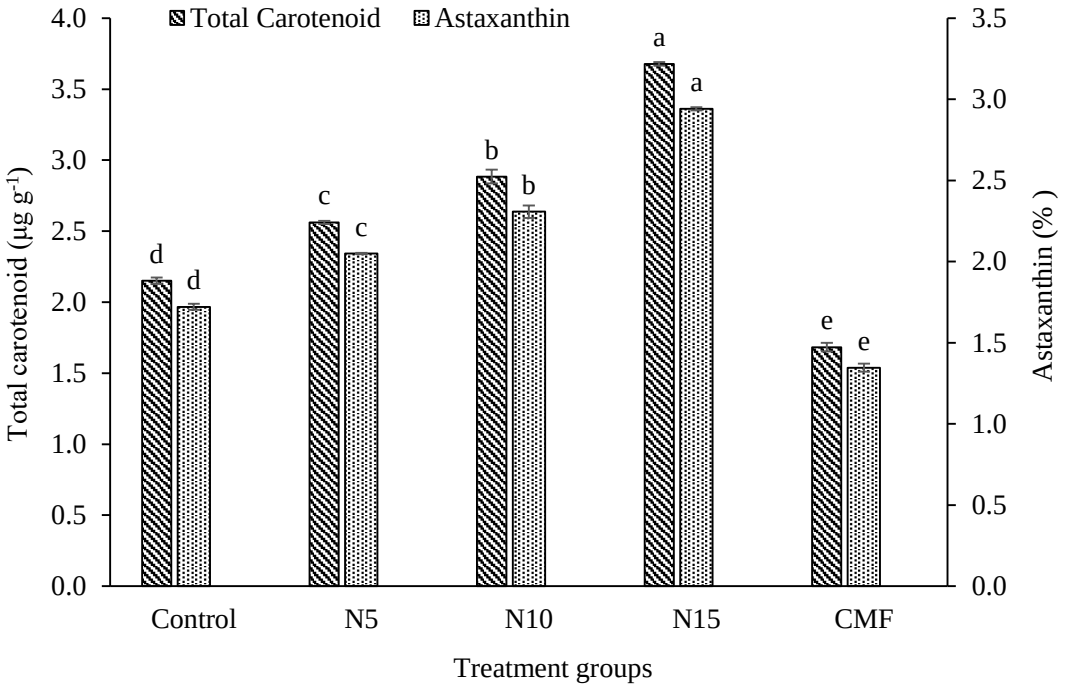


Figure 4.4 Total carotenoid and astaxanthin concentration of fin and muscle in guppy.

4.5 Oxidative stress index

4.5.1. Lipid peroxidation

Lipid peroxidation was measured by determination of malondialdehyde (MDA) as an end product of lipid peroxidation. In this study, the lipid peroxidation in guppy showed to have a significant ($P < 0.05$) difference among treatments. Figure 4.5.1 represents the changes of lipid peroxidase. The amount of malondialdehyde recorded in the control and N5 group were 3.729 ± 0.01 nmoles g^{-1} and 3.703 ± 0.01 nmoles g^{-1} and showed no significant ($P > 0.05$) differences. Whereas, the results obtained from N10 and N15 groups were significantly lower ($P < 0.05$) than the control group (3.385 ± 0.01 and 3.338 ± 0.02 nmoles g^{-1} respectively). Lipid peroxidation level was marginally higher in the CMF than in all other groups (4.095 ± 0.01 nmoles g^{-1}).

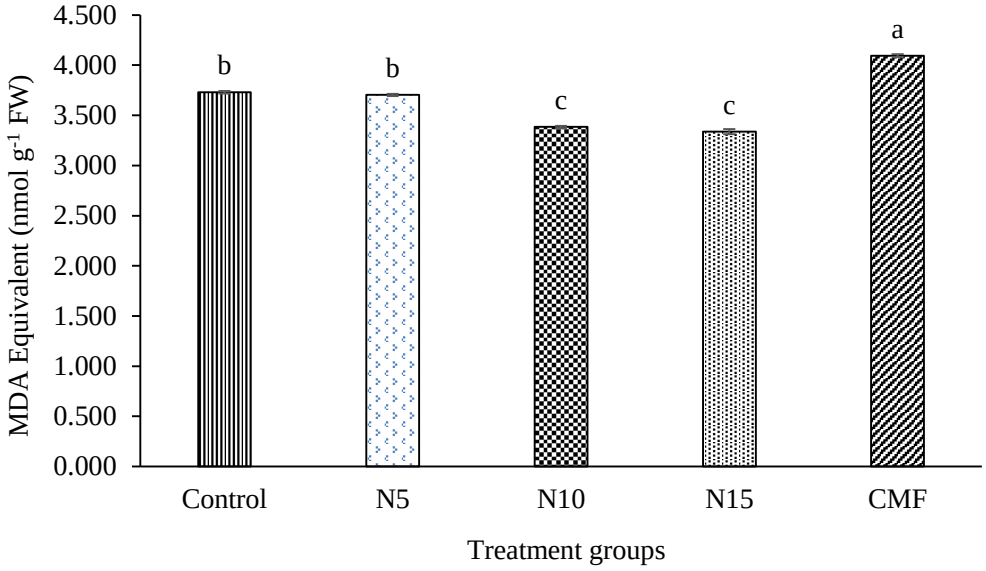


Figure 4.5.1 Level of lipid peroxidation found in guppy fed with formulated diets after 100 consecutive days of culture period

4.5.2. Hydrogen peroxide (H_2O_2) content

Changes in H_2O_2 content are shown in Figure 4.5.2. Fish fed with the addition of *Nannochloropsis* sp. in their diet showed significantly ($P < 0.05$) lower H_2O_2 compared to CF (Figure 4.5.2). The H_2O_2 content in fish fed with 15% (7.83 ± 0.2 mM g^{-1} FW), 10% (8.7 ± 0.08 mM g^{-1} FW) and 5% (8.93 ± 0.1 mM g^{-1} FW) algal inclusion diets, significantly ($P < 0.05$) decreased compared to control and CMF. Whereas, there was no significant differences ($P < 0.05$) found between N5 and N10 groups. The H_2O_2 content in control group was 10.23 ± 0.2 mM g^{-1} FW. The results also revealed that, commercial feed fed groups showed to have highest H_2O_2 (12.43 ± 0.8 mM g^{-1} FW) content than the other groups.

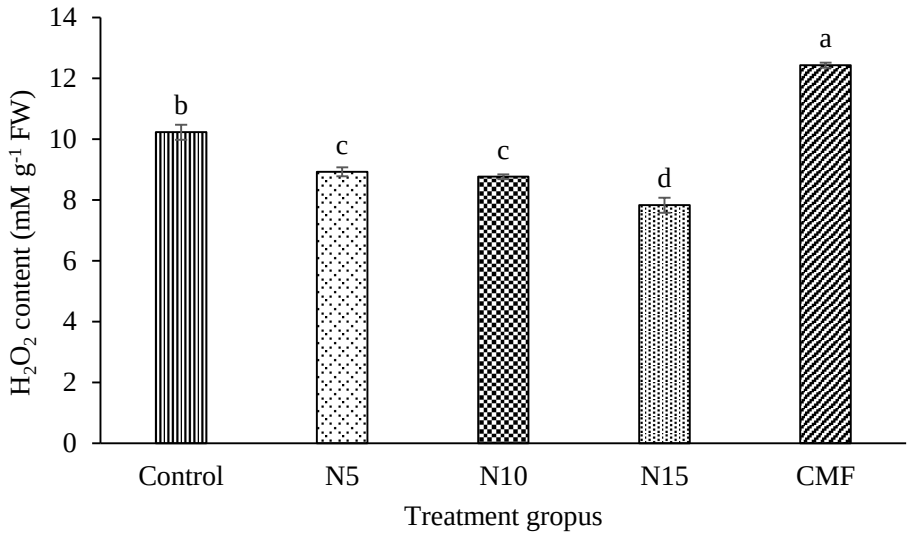


Figure 4.5.2 Hydrogen peroxide content of guppy fed with formulated diets after 100 days of culture period

4.6. Total fry production

The highest fry production was obtained with 15% (96 number of fries), followed by 10% (83 number of fries), and 5% (65 number of fries) *Nannochloropsis* sp. inclusion feed compared to the control diet (61 number of fries) (Figure 4.6). The commercial feed fed group never bred throughout the whole experimental period. Total fry production was also calculated on a weekly basis. The number of fry produced weekly showed that females fed with 15% microalgae inclusion diet started earlier breeding (7^{th} week), producing a larger number of fry than the other treatment groups. N10, N15 and control groups started breeding in the following 8^{th} and 9^{th} weeks (Table 5).

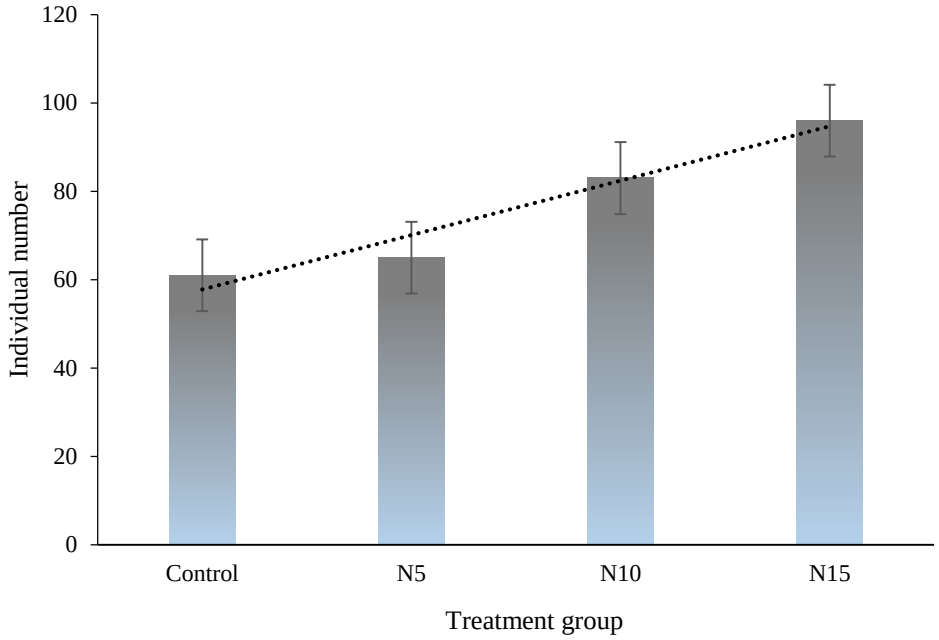


Figure 4.6 Total fry production number from each treatment group

Table 5 Mean weekly fry production (individual number) in different treatment groups.

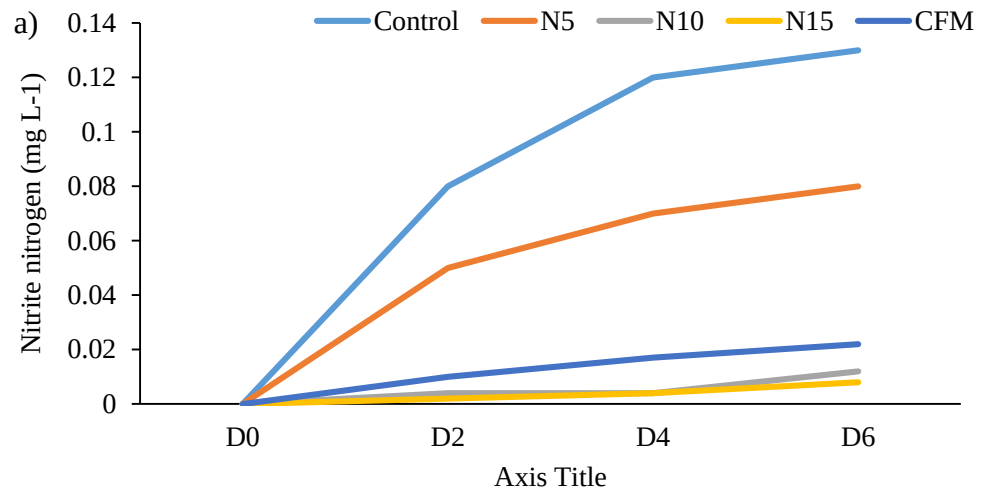
	Mean (weekly)						
Treatment	week-7	week-8	week-9	week-10	week-11	week-12	week-13
Control	0	0	2	5	4	3	0.25
N5	0	0	5	9	5	2	0
N10	0	6	9	3	5	0	0
N15	2	10	8	5	1	1	0

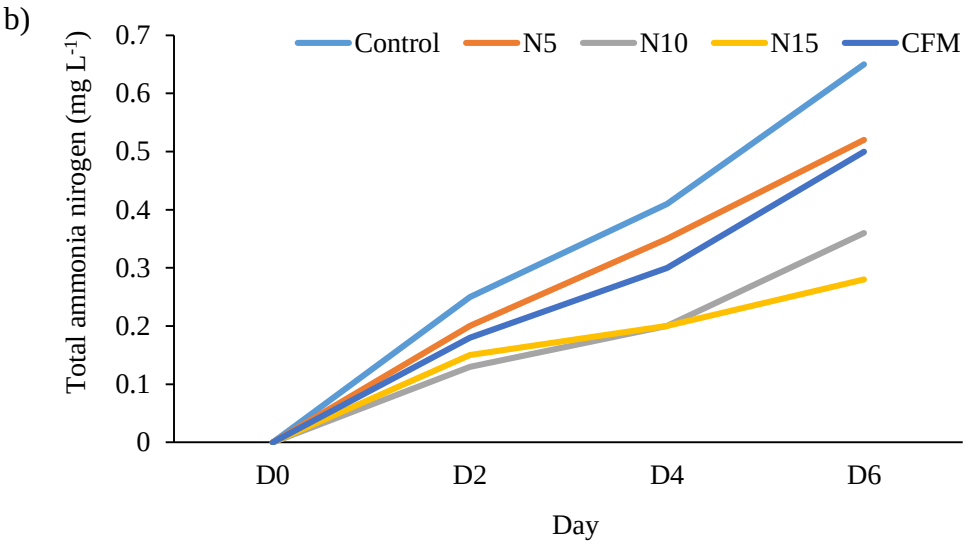
4.7. Physical and chemical parameters of culture water

In fish hatchery, increased TAN and NO_2-N level with culture time are important factors that affect the survival, health and growth performance of fish larvae. In this study, the inclusion of *Nannochloropsis* sp. into the feed leads to the improvement and maintenance of good water quality throughout the culture of guppy. Significantly lower NO_2-N (Figure 4.7a), TAN (Figure 4.7b) and PO_4-P (Figure 4.7c) were found in water samples from the tank of guppy supplemented with *Nannochloropsis* sp. compared to

a)

control tank. Furthermore, there was no significant difference in the physical parameters (temperature, DO and pH) of the water for all treatments during the whole experimental period (Table 6).





c)

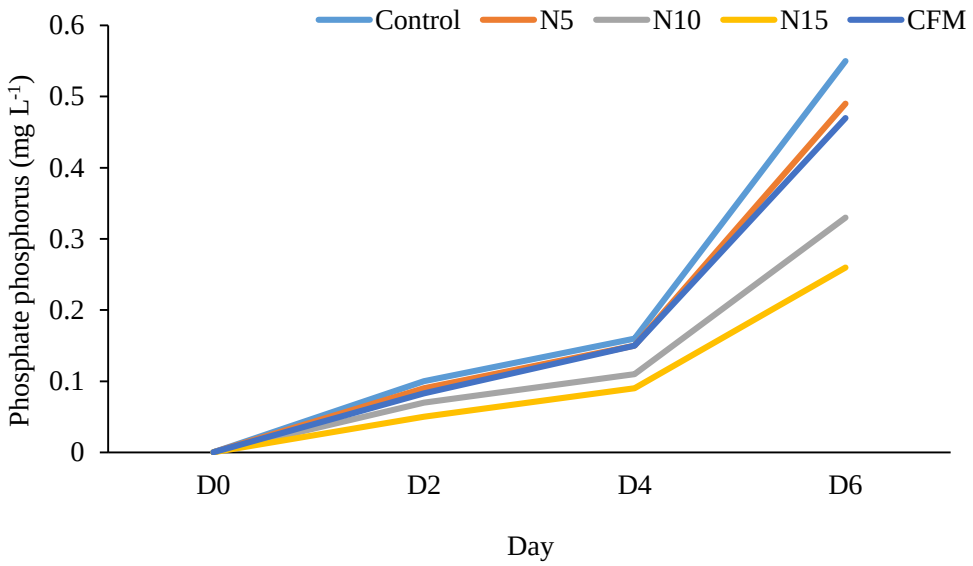


Figure 4.7: a. Nitrite nitrogen, b. total ammonia nitrogen, c. phosphate phosphorus concentration of the water in tanks with different diet treatment groups

Table 6 Temperature, dissolved oxygen and pH in water from control and treatment tanks

Treatment	Parameter		
	Temperature (°C)	DO (mg L^{-1})	pH
Control	26.92 ± 1.6[a]	6.03 ± 0.7[a]	8.24 ± 0.09[a]
N5	26.94 ± 1.2[a]	5.98 ± 0.6[a]	8.31 ± 0.07[a]
N10	27.03 ± 0.9[a]	6.12 ± 0.3[a]	7.92 ± 0.14[a]
N15	26.97 ± 1.5[a]	5.97 ± 0.6[a]	8.15 ± 0.10[a]
CMF	27.12 ± 1.2[a]	6.05 ± 0.5[a]	7.89 ± 0.08[a]

Data shown are mean ± SE (n = 3) for the whole 100 days of culture period. Means with the same letters are not significantly different (P > 0.05)

Chapter-5: Discussion

Alternative feed supplements of plant origin in enhancing the growth performances and coloration of ornamental fish have been prioritized in the progression of aquaculture industry for many years (Macias-Sancho et al., 2014; Cao et al., 2018). Microalgae are primary producers and potential sources of essential fatty acids and pigments that are transferred through the food chain to higher aquatic animals (Nath et al., 2012; Novoveská et al., 2019). The benefits of different microalgae as dietary supplements for Guppy fish have been demonstrated in several investigations (Dernekbasi et al., 2010; Dagar et al., 2010; Nath et al., 2012; Sarathchandra et al., 2018). The present study is the first to evaluate the use of *Nannochloropsis* sp. as a supplement to guppy (*Poecilia reticulate*) diets based on its potential in previous studies (Lubi´an et al., 2000; Sørensen et al., 2017; Lupatsch and Gbadamosi, 2018; Guimarães et al., 2021).

5.1. Growth performance

In this 14-week feeding experiment, all of the test diets were well accepted by the fish in all treatment groups. Iso-nitrogenous and isoproteic feed were formulated in the laboratory to perform the experiment (Vizcaíno et al., 2018; Seong et al., 2021). The microalgae inclusion levels of 5%, 10% and 15% were determined based on the results of previous studies on *Nannochloropsis* sp. feeding trials in European sea bass, Atlantic salmon and Atlantic cod (Walker and Berlinsky, 2011; Sørensen et al., 2017; Valente et al., 2019; Gong et al., 2020). The algal inclusion diet did not result in any significant changes in terms of growth rates or voluntary feed intake. However, the commercial feed fed groups showed a significant reduction in growth performance compared with the others (Vizcaíno et al., 2018). The values of average daily gain (mg) and SGR did not significantly differ among the feeding groups (Table 3). The N15 was found to have a significantly lower growth rate. Kiron et al. (2012) also reported that there was no change in the weight gain of Atlantic salmon (*Salmo salar*) fed diets with algal inclusion. The result is slightly dissimilar to that of Sørensen et al. (2017), who showed that *Nannochloropsis* sp. inclusion of more than 15% tends to result in lower weight gain and SGR. But some researchers established that, in case of algal feed formulation, the growth rate is optimum when partial replacement of dietary components is done up to 10 to 15% of the total feed content, depending on the species and their feed utilization efficacy (Sørensen et al., 2017; Valente et al., 2019). There was a slight but not

substantial difference in length increment, condition factor and FCR value among the control and microalgae inclusion diet fed groups, except for the commercial feed fed group. Similar to the other growth parameters, length increment and condition factor were found to be lower in the commercial feed fed groups. The growth performance results obtained from this study are consistent with the previous investigation performed on *Nannochloropsis*-fed to other species- European sea bass, Senegalese sole, (Vizcaíno, 2018; Valente et al., 2019). The inclusion of *Nannochloropsis* sp. did not change the overall growth performance of guppy in relation to the control feed. Some researchers found that, the feed intake rate is higher with a reduced feed conversion ratio when fish are fed with *Nannochloropsis* sp. incorporated feed as there may be some growth factors present in marine microalgae and to compensate for the slight reduction in lipid and energy content in feed (Norambuena et al., 2015; Sørensen et al., 2017). Also, the cell wall of *Nannochloropsis* sp. does not hinder palatability or digestibility processes like Chlorella and doesn't have any negative impact on feed acceptance in fish (Sørensen et al., 2017; Lupatsch and Gbadamosi, 2018). Another factor is that guppys are omnivorous fish. That's why they have greater amylase activity, which can contribute to improving the digestion efficiency of algae (Bjerkeng et al., 1999).

5.2. Survival and oxidative stress

Goiris et al. (2012) and Assunção et al. (2017) have found that microalgae contain key antioxidant compounds. Its bioactive substances, such as carotene, amino acids, polyunsaturated fatty acids, and others, play a major role in this. Algal dietary inclusion has also been associated with enhanced stress response, starvation tolerance, and health in fish (Güroy et al., 2011; Vizcaino et al., 2014; Norambuena et al., 2015). Even though a larger concentration of microalgae in feed will increase the nutritional and antioxidant content, fish can only tolerate a particular quantity of microalgae inclusion in diet (Abdu et al., 2017). This is because increasing the level of microalgae in the feed resulted in an increase in fiber content as well as a decrease in digestibility and palatability (Sudaryono et al., 1996).

In this investigation, the inclusion of *Nannochloropsis* sp. into the diet has shown a significantly higher survival compared to the groups fed with control and commercial feed after 100 days of culture period (Figure 4.2). The excessive production of reactive

oxygen species (ROS) and the preservation of the cell membrane by antioxidants against the production of free radicals are directly related to the performance and health of aquatic organisms (Mourente et al., 2002). The increased survival rate of guppy fed with *Nannochloropsis* sp. was observed in this study can be correlated to the levels of hydrogen peroxide (H_2O_2) and lipid peroxidation in fish tissue, which act as biomarkers of oxidative stress.

Guppy fed with the addition of *Nannochloropsis* sp. in their diet showed lower H_2O_2 compared to CF (Figure 4.5.2). The H_2O_2 content in fish fed with N15 was lowest than the other groups. The other *Nannochloropsis* sp. added feed fed groups (N10 and N5) showed significant decrease compared to control and CMF. This indicates that including up to 15% of *Nannochloropsis* sp. in the diet has a protective impact against oxidative stress in guppy under both stressful conditions and during growth, protecting the cell by lowering the content of H_2O_2 in tissue. Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) and can represent a risk for the cell (Cemeli et al., 2009). Furthermore, DNA oxidation is mostly generated by ROS and is a prevalent type of DNA damage (Azqueta et al., 2009) that must be decreased in the animal body. The findings of this study agree with those of Amar et al. (2004), who found that an aquatic organism's antioxidant defense is influenced by dietary variables. The necessity for natural antioxidant enzymes (such as catalase and superoxide dismutase) in scavenging hydrogen peroxide (H_2O_2) and superoxide radicals is reduced when dietary antioxidant supplementation is high (Lygren et al., 1999). According to studies by Sheikhzadeh et al. (2012) and Da Silva et al. (2015), supplementation of the high astaxanthin *H. pluvialis* into diet also leads to higher performance with increased antioxidant activity in juvenile *L. vannamei*. Other microalgae such as *Tetracelmis chuii* has been proved to be effective in lowering the oxidative stress in shrimp muscle (Abdu et al., 2017).

In accordance with the study by Tirmenstein and Reed (1988), during exposure to stress, lipid peroxidation can cause subcellular organelles and biomembrane damage. Some studies revealed that, lipid peroxidation (LPO) levels were not increased in animals fed with algal biomass. The increase of general antioxidant capacity brings benefits to fish health and can act as good agents in the prevention or treatment of environmental pollutants reducing oxidative damage (Abdelkhalek et al., 2015; Sayed et al., 2018).

In this experiment, LPO was assessed by determining malondialdehyde (MDA) as a final product of lipid peroxidation. MDA is an oxidative stress measure that reveals the degree of lipid peroxidation in membranes, which reflects cell and organelle integrity (Buege and Aust, 1978). The lipid peroxidation levels in the *Nannochloropsis* sp. feed fed groups were marginally lower than the control and CMF groups, respectively. This clearly shows that the microalgal antioxidant molecules were able to effectively reduce reactive oxygen species and protect the cells in the treatment groups (N15, N10, N5), but the control and commercial groups had a lesser protective impact. This could be due to the degradation of bioactive molecules in commercial fish feed. Hence, microalga-based diet contributed to enhance the defense mechanisms of fish than the other diets. In turbot (Qiao et al., 2019) and Atlantic salmon (Sørensen et al., 2017), *Nannochloropsis* sp. has also been linked to reduced lipid peroxidation and increased antioxidant capacity. According to Qiao et al. (2019), dietary *Nannochloropsis* sp. significantly reduced the deposition of MDA in the serum and liver. Microalgae also prevented lipid peroxidation in giant yellow croaker, *Pseudosciaena crocea*, fed with astaxanthin and *Haematococcus pluvialis*, compared to control diets without microalga supplementation (Li et al., 2014). Similarly, *Salmo salar* fish given the macroalga *Palmaria palmate* showed lower levels of lipid peroxidation than fish fed a control diet that did not include seaweed (Wan et al., 2016). Furthermore, antioxidants (b-carotene, astaxanthin, and lutein) extracted from the microalgae *Spirulina platensis*, *Haematococcus pluvialis*, and *Botryococcus braunii*, respectively, can significantly reduce lipid peroxidation levels in mammalian species (male Wistar rats) by scavenging hydroxyl radicals and free radicals (Ranga Rao et al., 2010).

Another antioxidant molecule found in algae is chlorophyll; these photochemically active chemicals have been linked to a variety of health advantages, including antimutagenic, antigenotoxic, and antioxidant qualities (Antonio and Roca, 2020). Despite the antioxidant properties, there are just a few research on the impact of carotenoids on DNA repair (Collins, 2001; Krinsky and Johnson, 2005; Azqueta and Collins, 2012). *Nannochloropsis* sp. is high in carotenoids, which can absorb free radical energy and prevent oxidative damage to tissues. After exposure to mercury chloride, the dietary effect of carotene supplemented feed was favorable to toxicity reduction through regulating immunological and antioxidant functions in Nile tilapia

(Elseady and Zahran, 2013). *Haematococcus pluvialis* in fish feed has been proved to improve the antioxidant system and some biochemical parameters (Sheikhzadeh et al., 2012). Antioxidant enzymes were more readily accumulated and biologically active compared to fish fed on basal diet. The adaptive response to prevent lipid peroxidation and cell injury in fish muscle was triggered by enhanced antioxidant enzyme production. These findings support our hypothesis that *Nannochloropsis* biomass supplementation has a beneficial effect due to physiologically active chemicals, particularly carotenoids.

5.3. Nutritional Composition

No significant difference was observed in protein content among the experimental groups fed with control and other test diets. These results are similar to those reported in other reaches (Sørensen et al., 2017; Vizcaíno, 2018; Gong et al., 2020) suggesting that dietary inclusion of microalgae in feed does not affect digestible nitrogen intake and gain, modifying body protein content (Batista et al., 2020). But the result/value of carcass lipid content was found to be dissimilar from the previous reports. But total fatty acid content was reported to be rich in shrimp tail muscle (Ju et al., 2009) which may be in line with this current finding.

The total lipid content of guppy was higher in groups fed with test diets compared with the control one. This may be because all the test diets were iso-lipidic and the apparent digestibility coefficient (ADC) of *Nannochloropsis* sp. is high in lipids and eicosapentaenoic fatty acid (Guimarães et al., 2021). A previous investigation on lipid availability of different marine microalgae have suggested that, *Nannochloropsis* sp. contains high amount of lipid levels (16.6 percent) and can accumulate high levels of polyunsaturated fatty acids (Lupatsch and Gbadamosi, 2018). Also the proximate composition can differ with different life stages of the fish and also influenced by feeding frequency, feed composition and environmental parameters like exogenous factors (Shearer, 1994). These might be the reasons why the algal inclusion diet fed groups retained more lipid in their bodies.

The carbohydrate content of the commercial diet was higher, whereas there was no significant difference found in the other treatment groups, which is similar to the findings reported by Vizcaíno (2018). It should be also noted that the nutrient retention values couldn’t be discussed here as the data on proximate composition of initially

stocked fish was not recorded which might provide some valuable information about nutrient retention efficiencies of fish fed with *Nannochloropsis* sp. inclusion diet. Also we couldn't analyze the fatty acid profile of both the test diets and experimental fish after the termination of the trial.

5.4. Total carotenoids

Fish species need dietary supplement rich in carotenoid to enhance their body coloration because these contents can't be synthesized de novo (Gupta et al., 2015). Results from this study showed that, an increase in inclusion level of *Nannochloropsis* sp. elevated the total body coloration of the experimental fish. The highest pigment concentration was observed in group fed with 15% *Nannochloropsis* sp. inclusion diet. In this experiment, whole cell biomass of *Nannochloropsis* sp. was used as a feed supplement. Flores et al. (2008) suggested that, whole algae biomass inclusion results in better pigmentation effects. The pigment concentration was higher in test diets (N15, N10 and N5 respectively). On the other hand, females and males of *P. reticulata* displayed less skin coloration when fed with commercial feed compared with the other treatment diets. Although the commercial feed supplied in this experiment had a high protein value, it didn't contribute to increase the color intensity of the target fish. The possible explanation of this outcome could be that, the experimental feeds contained higher pigment concentrations added from microalgae cells incorporated into those diets. Whereas, the commercial feed is formed primarily for the purpose of growth and low cost, so it doesn't contain any additional components that trigger the skin pigmentation of fish.

Whereas, along with many beneficial compounds, *Nannochloropsis* spp. are able to build up a high concentrations of pigments such as astaxanthin, zeaxanthin and canthaxanthin (Lubi´an et al., 2000; Islam et al., 2021). As compared with many other microalgae, *Nannochloropsis* sp. contains a significantly higher concentration of carotenoids. Also, the carotenoid compounds present in *Nannochloropsis* spp. are easily absorbed and converted into usable pigments as they are highly assimilable by animals (Guimarães et al., 2021). According to some researchers, the amount of pigment deposition in integument increases with the concentration of carotenoids in the diet (Sommer et al., 1992). The addition of *Nannochloropsis* sp. in feed positively increased the level of astaxanthin content in shrimp tail muscles. (Ju et al., 2009).

5.5. Reproductive performance

Broodstock must be fed a good-quality diet for several months before spawning (Rainuzzo et al., 1997). In this feeding trial, dietary algae inclusion as a replacement of fish meal resulted in a significant increase in reproductive performance (total fry production) of guppy. The highest relative fecundity was obtained with the diet containing *Nannochloropsis* sp, whereas the fry production rate was lower in control and commercial feed fed groups, which gave birth to premature fries (only two of them were born as healthy fry). This study is the first to observe the effect of dietary *Nannochloropsis* sp. inclusion feed on the breeding status of guppy or any other fish. As stated before, *Nannochloropsis* spp. contains some essential molecules for reproduction, such as EPA (Eicosapentaenoic acid), one of the several omega-3 fatty acids. Together with protein, lipid levels in the bloodstock diet are essential for early gonadal development, efficacious reproduction and survival of offspring (James and Sampath, 2004). The fatty acids are important sources of energy during ovulation and early embryonic development (Sargent, 1995; Rainuzzo, 1993). This n-3 HUFA enhanced the reproduction performance of female rainbow trout and white bass (*Morone chrysopes*) in freshwater (Lane, 2005). Another possible factor for the increased reproductive rate could be the guppy's improved body coloring. The carotenoid content obtained through dietary sources deposits in male's skin tissue and signifies its mating quality to the female (Chatzifotis et al., 2005). In case of many fishes, including guppys, studies have revealed that body pigmentation can significantly affect the breeding performance of fish (Endler, 1980) that can be obtained only through dietary sources.

5.6. Effect of feeding with *Nannochloropsis* sp. on water quality of culture tank

In commercial fish hatchery and other culture systems, increased TAN and NO_2-N level with culture time are important factors that affect the survival, health and growth performance of fish larvae and juvenile (Chin and Chen, 1987). In this study, the inclusion of *Nannochloropsis* sp. into the feed leads to the improvement and maintenance of good water quality throughout the culture period. Significantly lower NO_2-N (Figure 4.7a),), TAN (Figure 4.7b) and PO_4-P (Figure 4.7c),) were found in water samples from the tank of PL supplemented with *Nannochloropsis* sp. compared to control during the culture. This result was in accordance with Guedes and Malcata

(2012), who showed that microalgae could stabilize and improve the water quality of culture. Although dried microalgae were used in this study, there are still some viable microalgal cells present even if it is at a very low level (Day, 2007). Thus, there is a possibility of microalgal cells from leftover feed to start growing again in the tank. Throughout the guppy culture period, a slow increase in green water concentration in the tank was observed. It was contributed by the increase in microalgae concentration which absorbs the nutrients available, especially in tank N15, N10 and N5. In another study, Chen et al. (2012) found that, *Tetraselmis chuii* showed the highest TAN uptake which led to lower nutrient toward the end of culture compared to the other microalgae species studied. These results are also similar with those to the findings from Abdu et al. (2017). Thus, minimal water exchange can be considered while rearing guppy in large scale fed with *Nannochloropsis* sp. inclusion which is beneficial due to the cost and labor usually needed for frequent water exchange in aquaculture to maintain a good water quality (Thompson et al., 2002). Meanwhile, there was no significant difference in the physical parameters (temperature, DO and pH) of the water for all treatments during the whole experimental period (Table 5).

Chapter-6: Conclusions

In this investigation, *Nannochoropsis* showed to be a promising source of pigment and nutritional components required for fish growth and reproduction. Guppy fed with *Nannochloropsis* incorporated feed showed enhanced body coloration and earlier reproductive performance. It was also found that, adding the whole *Nannochloropsis* sp. biomass as a modest-level (close to 15%) feed additive to a control diet can significantly improve Guppy's overall performance without any negative effects on their growth in comparison with the control feed fed groups. The study also confirmed that a diet containing optimal microalgal biomass supplement may play a great role in protecting fish from the increased production of reactive oxygen species and oxidative stress damage, thus enhancing disease resistance and survival.

In conclusion, it can be said that the microalgal ingredient used in our fish feed has the potential to become an alternative dietary ingredient to be used in organic feed production with a stable and affordable supply of healthy protein and oil without causing harm to oceans or food security of resource-poor people. Furthermore, this preliminary study provides support for future research aiming at optimizing the concentration of microalgal biomass in the diet, the period of carotenoid supplementation, and identifying the potential of bioactive compounds present in *Nannochloropsis* sp. and their effects on different biological factors of other species used in aquaculture.

Chapter 7: Recommendations and Future perspectives

For microalgae to become a successful alternative to fish meal, it has to overcome a number of obstacles. Similar to many alternate feeds, the microalgae-based fish feed has a low palatability, but this can be improved by changing the texture of the feed and adding attractants/ stimulants that fish's chemosensory systems accept. Some microalgae have lower protein content and higher carbohydrate content than conventional feed- reducing feed suitability. Thus, careful species selection and evaluation of growth in various environments are required to formulate suitable microalgal feed and reduce the production cost. In many cases, when compared to fish meal, the microalgal feed resulted in lower fish intake. However, if ingredients like taurine are added to microalgal feed, fish will have better intake, resulting in superior growth performance. Production systems, harvesting, and processing technologies are yet to be optimized at large scales. In the future, innovative manufacturing in feed combined with novel upstream and downstream processing technologies for microalgae biomass production can effectively replace fish meal and provide a sustainable solution. Therefore, future research attempts may include the followings:

i. Identification, isolation and screening of more potential indigenous microalgae from both freshwater and coastal area of Bangladesh
ii. Assessment of nutritional composition and other bioactive components found in different microalgae and their possible use in aquaculture
iii. Evaluation of the effect of microalgal based diet on growth, survival, proximate composition, breeding, coloration and other physiological properties of different commercially valuable fish and other aquatic species (crustaceans and mollusk)
iv. Comparative study of different commercially valuable fish fed with potential microalgae in multi-spectrum aspect.
v. Larval response to different microalgal feeding (growth and survival)
vi. Amino acid and fatty acid profiling of the culture species

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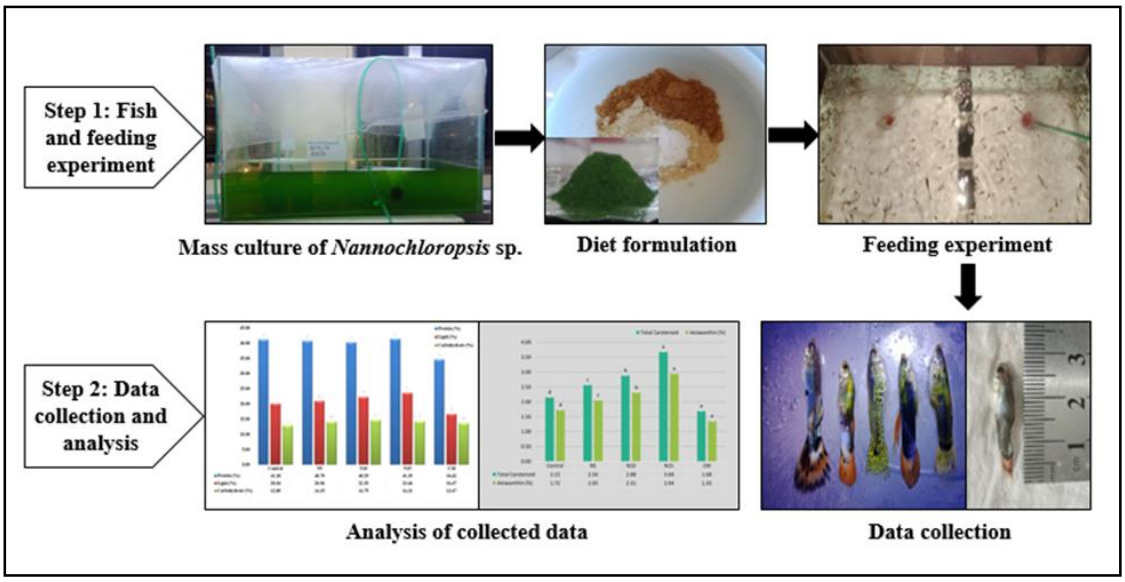
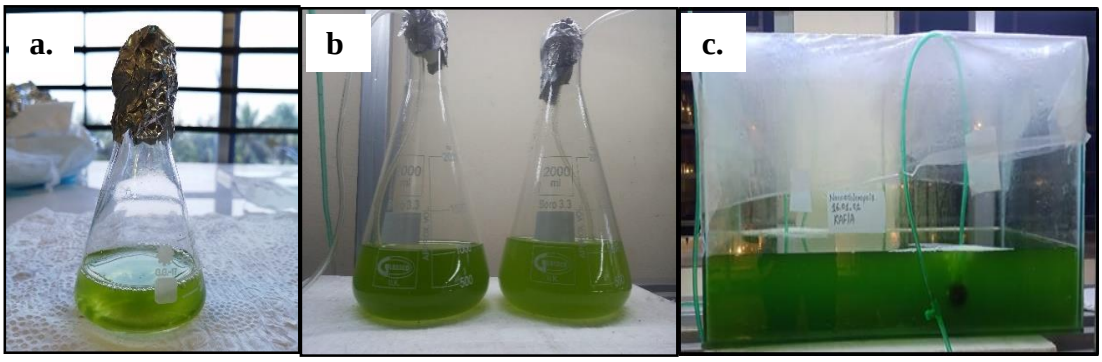
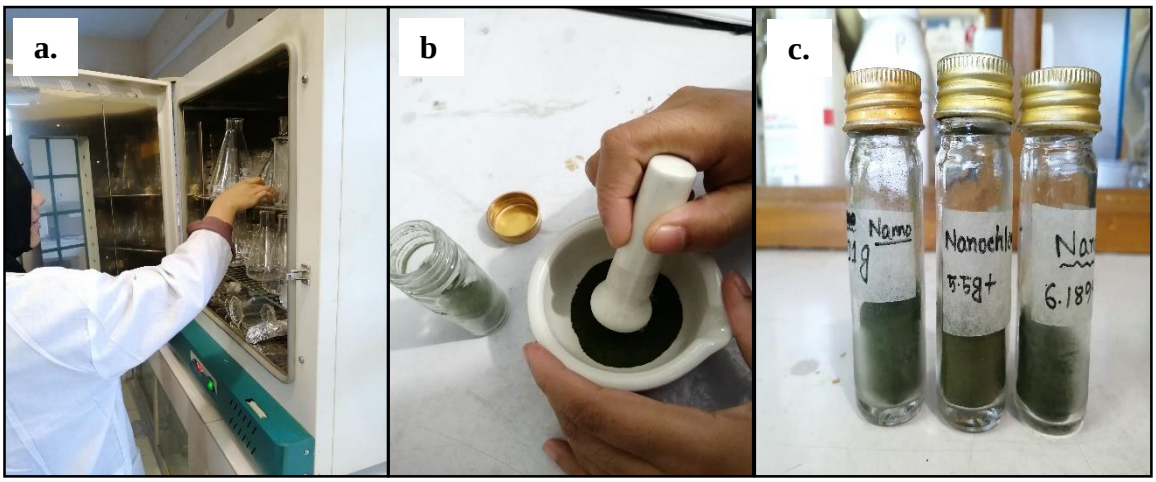
Appendices



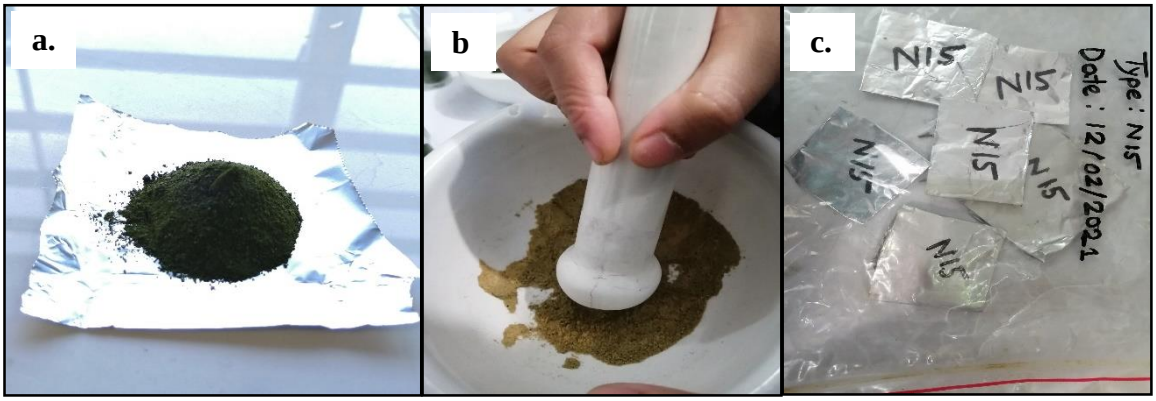
Appendix A: Brief overview of the activities related to the feeding trial (100 consecutive days) of guppy fed with *Nannochloropsis* sp. incorporated experimental feeds



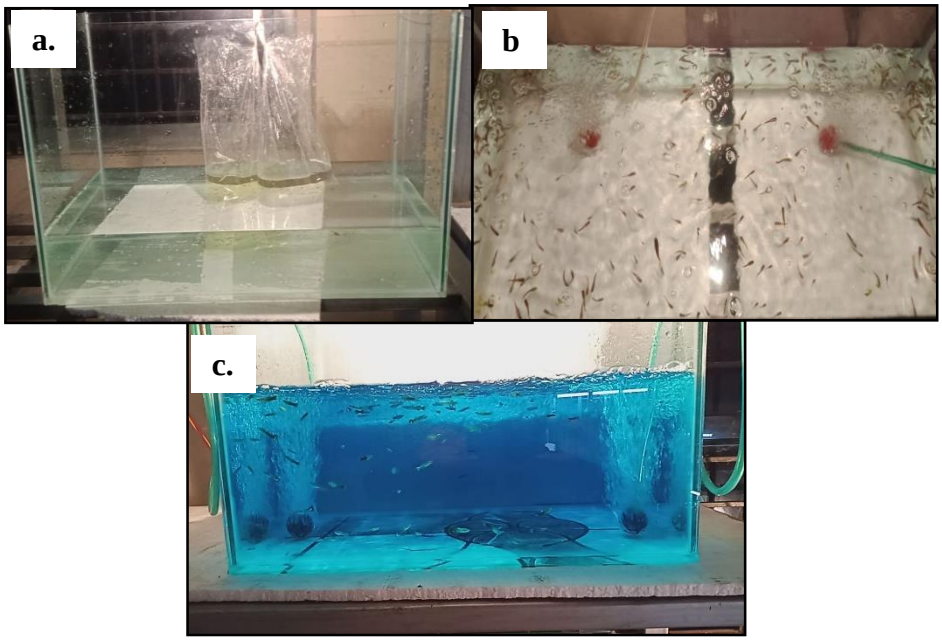
Appendix B: Culture of algal biomass **(***Nannochloropsis* sp.); a. collected pure isolates, b. Stock culture, c. mass culture in glass tank



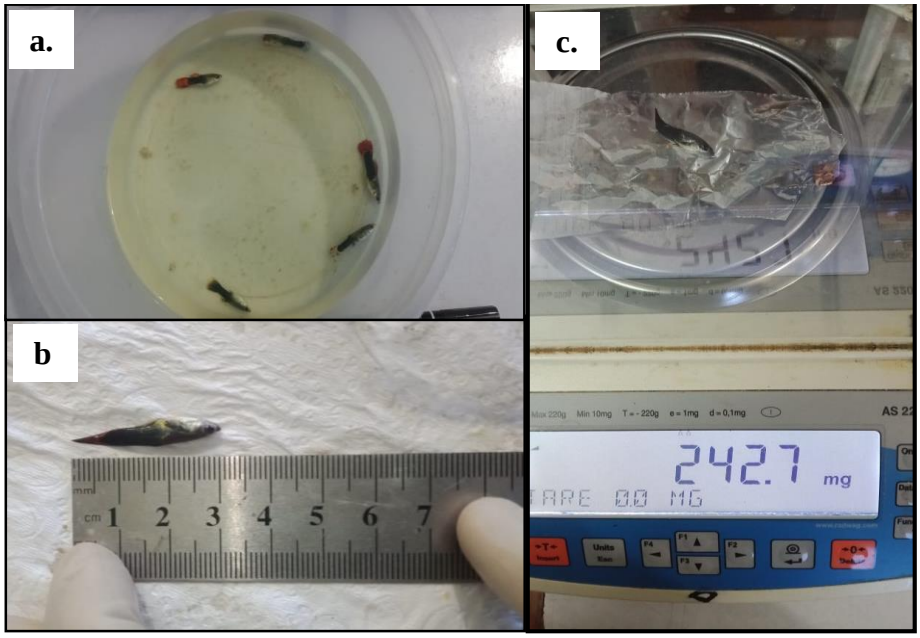
Appendix C: Harvesting *Nannochloropsis* sp. as dry algal biomass; a. oven drying, b. grinding, c. storing in air tight glass vials



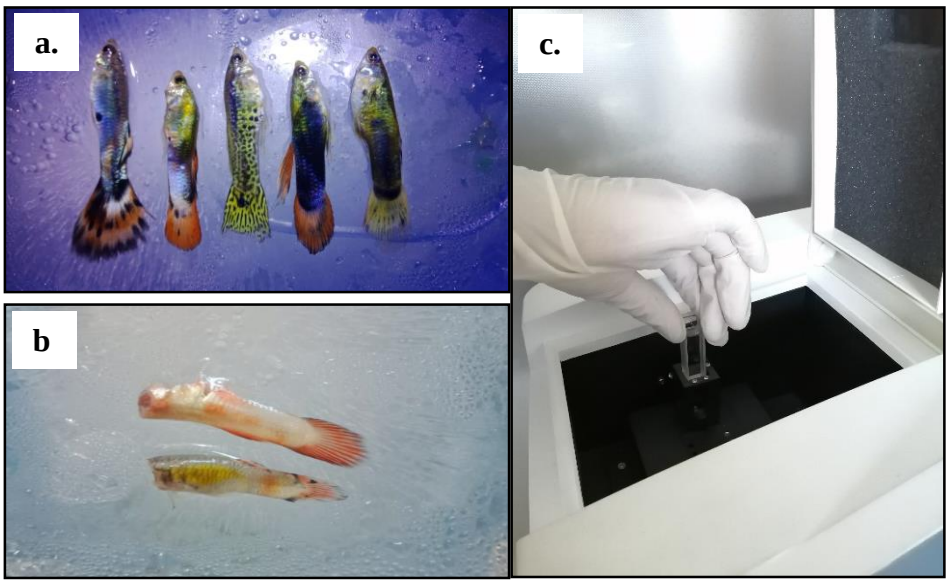
Appendix D: Test diets preparation; a. dry algae powder, b. grinding all the feed ingredients with morter and pestle, c. storing the prepared feed in in sealed plastic bags



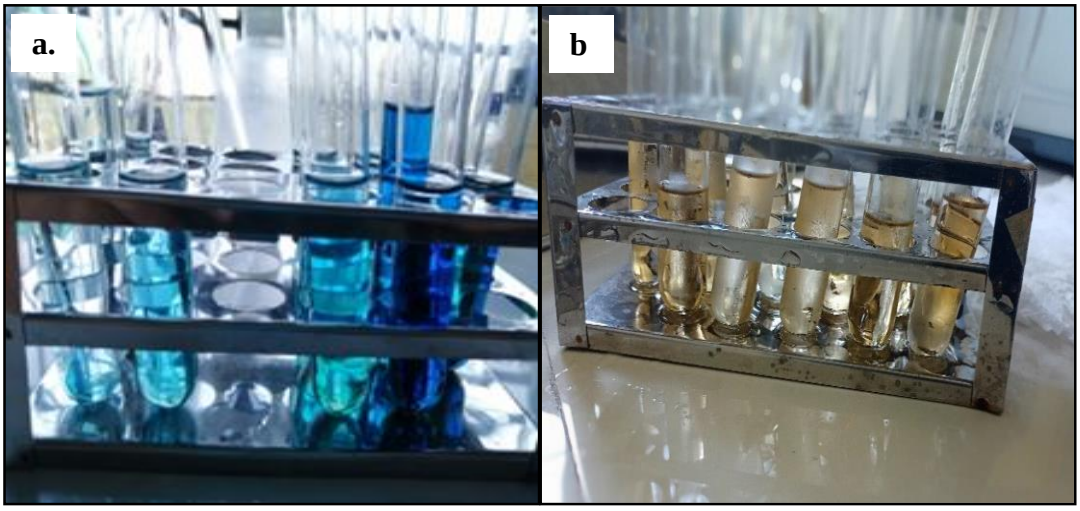
Appendix E: Pre-stocking and adaptation activities; a. conditioning with the water temperature, b. acclimatization with the laboratory conditions for three days, c. disinfecting the collected fries using $KMnO_4$



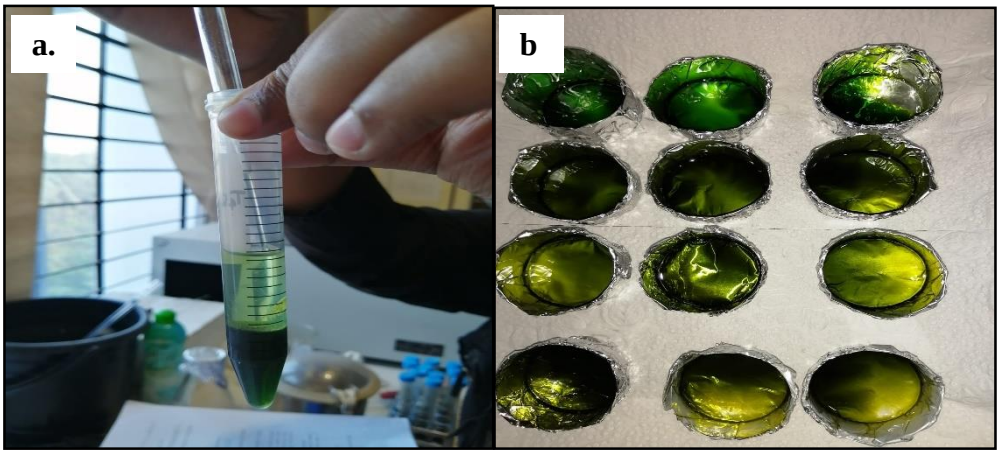
Appendix F: Assessment of growth performance; a. anesthetization with clove oil, b. length measurement, c. weight estimation



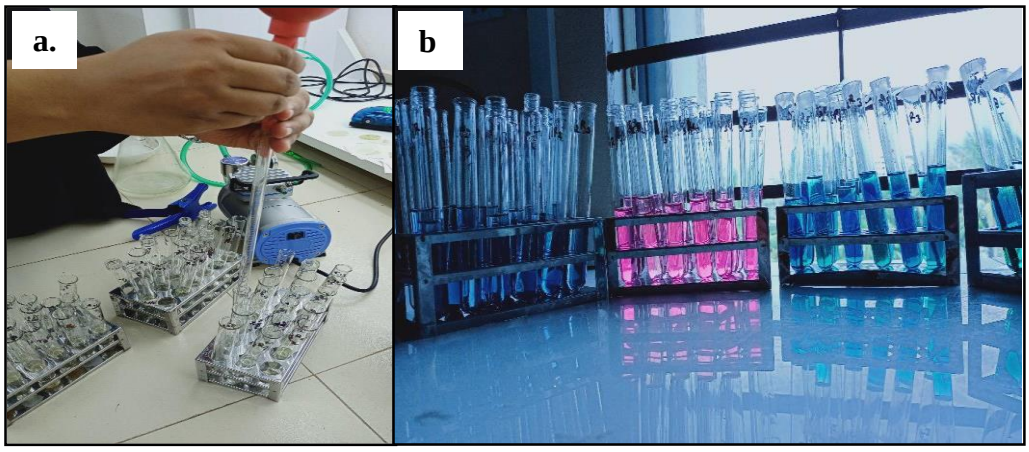
Appendix G: Determination of total carotenoids; a. collection of fish, b. beheading, c. measurement of optical density in spectrophotometer



Appendix H: Determination of a. protein and b. carbohydrate content



Appendix I: Determination of lipid content; a. separation of supernatant containing lipid, b. lipid containing aluminum plates



Appendix J: Determination of water quality parameters; a. addition of required reagents, b. test tubes containing final solutions

Appendix K: One-way Analysis of Variance Examining proximate composition (protein, lipid, carbohydrate), total carotenoid content, lipid peroxidation and hydrogen peroxidase activity, chemical parameters (Nitrite-N, total ammonia nitrogen, phosphate phosphorus) of culture water

ANOVA					
Protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37.932	4	9.483	6.489	.008
Within Groups	14.615	10	1.461		
Total	52.546	14			

ANOVA					
Lipid					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	65.264	4	16.316	5.424	.014
Within Groups	30.079	10	3.008		
Total	95.343	14			

ANOVA					
Carbohydrate					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.638	4	3.409	3.863	.038
Within Groups	8.826	10	.883		
Total	22.464	14			

ANOVA					
Carotenoid					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.491	4	1.123	11.012	.011
Within Groups	.510	5	.102		
Total	5.001	9			

ANOVA					
LPO					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.124	4	.281	527.323	.000
Within Groups	.005	10	.001		
Total	1.129	14			

ANOVA					
H_2O_2					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38.043	4	9.511	150.168	.000
Within Groups	.633	10	.063		
Total	38.676	14			

ANOVA					
Nitrite-N					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.026	4	.006	31.966	.000
Within Groups	.002	10	.000		
Total	.028	14			

ANOVA					
Total Ammonia Nitrogen					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.105	4	.026	1.184	.035
Within Groups	2.221	10	.022		
Total	10.326	14			

ANOVA					
Phosphate phosphorus					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.038	4	3.010	.265	.044
Within Groups	3.362	10	2.036		
Total	17.40	14			

Brief biography of the student

Razia Sultana is the second daughter of Md. Nurul Islam and Shahina Islam, was born in 25th April, 1997 in Dhaka, Bangladesh. She has achieved Secondary School Certificate from Ashadpur Haji Shiraj-Ud-Doullah High School and Higher Secondary Certificate from BCIC College. She has also achieved B.Sc. Fisheries (Hons) from Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University. She is now a candidate of Master of Science in Aquaculture under the Department of Aquaculture, Chattogram Veterinary and Animal Sciences University.

She was employed as a research assistant for a period of January 2020 to December 2021 in the project entitled "Aquaculture wastewater as a low cost medium for mass production of marine microalgae and it's utilization as feed for culturing sea bass and crab larvae" funded by Bangladesh Fisheries Research Institute (BFRI). She has already published some research articles in well reputed national and international journals. During COVID 19 severe pandemic (2020), she served the nation through working as a molecular biologist (Voluntary basis) at Chattogram Veterinary and Animal Sciences University, Chattogarm and Bhasabir M.A Wadud COVID 19 Detection Lab, Chandpur Medical College, Chandpur. From January 2022 to till now she is working in a project entitled "Isolation and identification of indigenous microalgae from different coastal regions of Bangladesh and its utilization as live feed for aquaculture industry" funded by Krishi Gobeshona Foundation (KGF) under Dr. Helena Khatoon. Her research interest includes ornamental fish (growth assessment, nutritional profile, coloration, breeding, disease, production rate etc.) and culture of microalgae. She is passionate to qualify herself as a competent researcher, and thus to develop the aquaculture sector of Bangladesh.

List of Publications

Publications	Status
Rahman MR, Islam SMR, Nayma Z, **Sultana R**, Sarker J. 2020. Bio-economic evolution of snakeheads and Indian major carps culture in IMTA system. Bangladesh Journal of Veterinary and Animal Sciences. 8 (1): 117-127.	**Published**
Khatoon H, Penz KP, Banerjee S, Rahman MR, Minhaz TM, Islam Z, Mukta FA, Nayma Z, **Sultana R**, Amira KI. 2021. Immobilized *Tetraselmis* sp. for reducing nitrogenous and phosphorous compounds from aquaculture wastewater. Bioresource Technology. 338: 125529.	**Published**
Khatoon H, Leng MY, Rahman MR, Sarker J, Minhaz TM, **Sultana R**, Nayma Z, Mukta FA. 2021. Efficiency of *Chlorella vulgaris* beads in improving water quality and growth of juvenile siamese fighting fish (*Betta splendens*). Bangladesh Journal of Veterinary and Animal Sciences. 9 (1): 74-86.	**Published**
Usha SZ, Rahman MR, Sarker J, Hasan SJ, **Sultana R**, Nayma Z, Mukta FA, Khatoon H. 2021. Cultivation of *Chlorella vulgaris* in aquaculture wastewater as alternative nutrient source and better treatment process. Bangladesh Journal of Veterinary and Animal Sciences. 9 (1): 43-51.	**Published**
Nayma Z, Khatoon H, Rahman MR, Mukta FA, **Sultana R**, Hossain MN, Iqbal MZ. 2021. A comparative study on the productivity of selected tropical freshwater microalgae. Bangladesh Journal of Fisheries. 33 (2): 255-264.	**Published**
Sultana R, Khatoon H, Rahman MR, Haque ME, Nayma Z, Mukta FA. 2022. Potentiality of *Nannochloropsis* Sp. As Partial Dietary Replacement of Fishmeal on Growth, Proximate Composition, Pigment and Breeding Performance in Guppy (*Poecilia Reticulata*). Bioresource Technology Reports. 18: 101112.	**Published**
Mukta FA, Khatoon H, Rahman MR, Acharjee MR, Newase S, Nayma Z, **Sultana R**, Hasan SJ. 2021. Effect of Different Nitrogen	**Accepted**

Concentration on the Growth, Proximate and Biochemical Composition of Freshwater Microalgae *Scenedesmus communis*. Journal of Energy and Environmental Sustainability. 11: 36-42.	
Nayma Z, Khatoon H, Rahman MR, Mukta FA, **Sultana R**, Hossain MN. 2022. A comparative study on the productivity of selected tropical freshwater microalgae. Journal of Innovation in Applied Research.	**Accepted**