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Assessment of novel algal biomass sources as potential ingredients in diets for tilapia (*Oreochromis niloticus*)

By

Benjamin Paul Eynon

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Assessment of novel algal biomass sources as potential ingredients in diets for tilapia (*Oreochromis niloticus*)

Benjamin P. Eynon

Abstract

The aquaculture sector is currently seeking alternative feed ingredients to ensure future socioeconomic and environmental sustainability; microalgae sources are seen to be a promising option.

Two nutrition trials were conducted in order to assess substituting plant based protein sources in diets for juvenile Nile tilapia (*Oreochromis niloticus*), with a range of inclusion levels of two proprietary defatted microalgae samples (*Nannochloropsis* sp. and *Chlorella* sp.). The experimental algal ingredients utilised were by-products sourced from a pilot-scale biofuel operation. In both trials all experimental algal diets, along with their respective algal free controls, were isonitrogenous and isoenergetic. Fish growth performance, feed utilisation, biological indices, body composition, haematological parameters, histological appraisal of liver and the gastro-intestinal tract and assessment of epithelial skin colouration were evaluated.

In trial 1, the final weight gain, specific growth rate, feed conversion ratio, biological indices and survival rate of fish were not affected by *Nannochloropsis* sp. supplementation ($p>0.05$). Carcass composition analysis indicated that fish *Nannochloropsis* (5%, 10% and 15%) had significantly lower body protein and lipid levels. Significantly elevated white blood cell counts, intestinal mucosal fold length and increased hepatocyte nuclei density were observed in fish fed the 15% inclusion. Using a novel photographic application method, 10% and 15% inclusion levels were observed to significantly modulate red spectrum pigmentation.

In trial 2, all growth performance, feed utilisation and biological indices investigated showed significant differences at 60% inclusion of *Chlorella* sp. ($p<0.05$) with no significant differences in survival compared to control diets. Carcass composition revealed 60% inclusion significantly lowered fish body protein and lipid content, with ash increasing. Significantly elevated granulocyte counts were discovered at 30% and 60% Chlorella sp. inclusion. All algal supplemented diets (15%, 30% and 60%) indicated a significant increase in mid-intestine surface area. Significant increase of hepatocyte nuclei density was also observed in fish fed the 60% inclusion. No amplification of skin colouration was observed in this second trial.

These studies indicate great potential for below 15% level inclusions of dried microalgae meal as a by-product from the biotechnology sector; particularly with regards to immunomodulation and promoting external pigmentation, applicable to commercial foodfish and ornamentals alike.

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List of Abbreviations

µl	Microliter		FM	Fish Meal
µm	Micrometre		FO	Fish Oil
ANFs	Anti-nutritional factors		g	Grams
ANOVA	Analysis of variance		g dl^{-1}	grams per decilitre
AOAC	Association of Analytical Communities		G3P	Glyceraldehyde 3-phosphate
ATP	Adenosine triphosphate		GIFT	Genetically Improved Farmed Tilapia
AU	Arbitrary Unit		Gm	Genetic Modification
BSE	Bovine spongiform encephalopathy		GM	Genetically modified
MC	Carboxymethyl cellulose		MT	Genetically male tilapia
CO$_2$	Carbon dioxide		H and E	Hematoxylin and eosin stain
CP	Crude Protein		Hb	Haemoglobin
DEFRA	Department for Environment Food & Rural Affairs		Hct	Haematocrit
DHA	Docosahexaenoic acid		HPLC	High-performance liquid chromatography
DPX	Dibutyl-phthalate xylene		HSI	Heptosomatic Index
EC	European Community		I D	Identification
EFSA	European Food Safety Authority		IFFO	The Marine Ingredients Organisation
EP	External Perimeter		IMS	Industrial methylated sprits
EPA	Eicosapentaenoic acid		I P	Internal perimeter
FAO	Food and Agriculture Organization		ISO	International Organization for Standardization
CR	Feed Conversion Ratio		K	Condition Factor
FL	Final Length		kJ g^{-1}	Kilojoule per gram
FL	Fold Length		LC-PUFAs	Long chain Polyunsaturated fatty acids

LM	Light microscopy	PUFA	Polyunsaturated fatty acids
M D	Microvilli density	RBC	Red blood cell
MJ	Mega Joules	RGB	Red, Green & Blue colour model
ml	Millilitre	rpm	Revolutions per minute
Mt	Metric tons	SCP	Single cell protein
N	Nitrogen	SD	Standard Deviation
NADPH	Nicotinamide adenine dinucleotide phosphate	SEM	Scanning Electron Microscope
NFE	Nitrogen-free extract	SGR	Specific Growth Rate
nm	nanometre	SOP	Standard Operating Procedure
NOAA	National Oceanic and Atmospheric Administration	t	Tons
		U V	Ultra Violet
PAPs	Processed animal proteins	TAGs	Triacylglycerols
PBR	Photobioreactor	W	Watts
PCV	Packed Cell Volume	WBC	White blood cell
P I	Protein intake	WG	Weight gain
PR	Perimeter Ratio		

Dedication

To Katie Jane,

My amazing wife and best friend,

whose sacrificial care for me and our two beautiful children,

made this achievement possible, Thank you.

Thank you, Finlay and Lottie-may for suffering my never ending homework,

"You must have finished by now?"

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I would like to thank Heliae®, in particular, Dr Eneko Ganuza, for supplying algal biomass for appraisal, making this research possible. I would also like to thank all the technical support staff that have taught me many new skills and techniques that will serve me well in the future. Thank you to the aquaculture group, which has helped and supported me during all those long sampling sessions and the completion of this thesis – Dr Anna Rodriles, Dr Mark Rawling, Dr Benedict Standon & Nathan Atkinson.

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Peter Russel, thank you for getting the ball rolling.

Authors Declaration

The author declares that the thesis describes original work that has not been previously presented for any other purpose and all views expressed herein are the opinion of the author

All experimental work involving animals was carried out in accordance with the Animals (Scientific Procedures) Act 1986 under Home Office legislation

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Chapter 1

Introduction

1.1 An overview of modern aquaculture

The current global population is already close to 7 billion and is projected to reach over 8 billion by 2030 and an estimated 9.5 billion by 2050 (United Nations, (2015). As the world population continues to grow, pressure will be placed upon arable land and freshwater reserves, as well as energy and biological resources. In particular, there will be a major requirement for high quality protein as a food source to sustain the expanding population; this will require a re-assessment of present agricultural practices in relation to conventional arable and livestock production (FAO 2012).

An increasing number of studies are emerging on the positive aspects of including fish as part of a well-balanced healthy diet, such as improved cardiovascular health, improved growth and development of children, along with general wellbeing and the maintenance of overall good health (Hostenkamp and Sorensen, 2010). This is due to fish being a valued source of protein, vitamins and minerals and especially omega-3 fatty acids (a key nutrient for brain and cognitive development) (Hamed *et al.*, 2015). Crawford *et al*. (1999) suggested that development of the human brain is linked to food sources rich in phospholipids, which contain long chain polyunsaturated fatty acids (PUFAs) (such as n-3 docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and n-6 (arachidonic acid (AA)), with *Homo sapiens* evolving not in a savannah habitat but in habitats rich in fish and shellfish resources.

During the 1950s and 60s the demand for fish was met by approximately 20 million tonnes of marine and inland captured fisheries (FAO, 2011). During the 1970s and 1980s, the average rate of increase for captured production slowed to approximately two percent per year, falling to almost zero in the 1990s. This decelerating of total captured fisheries could

be attributed to the general trend of the world's fishing areas reaching their maximum potential for captured fisheries (Figure 1.1).
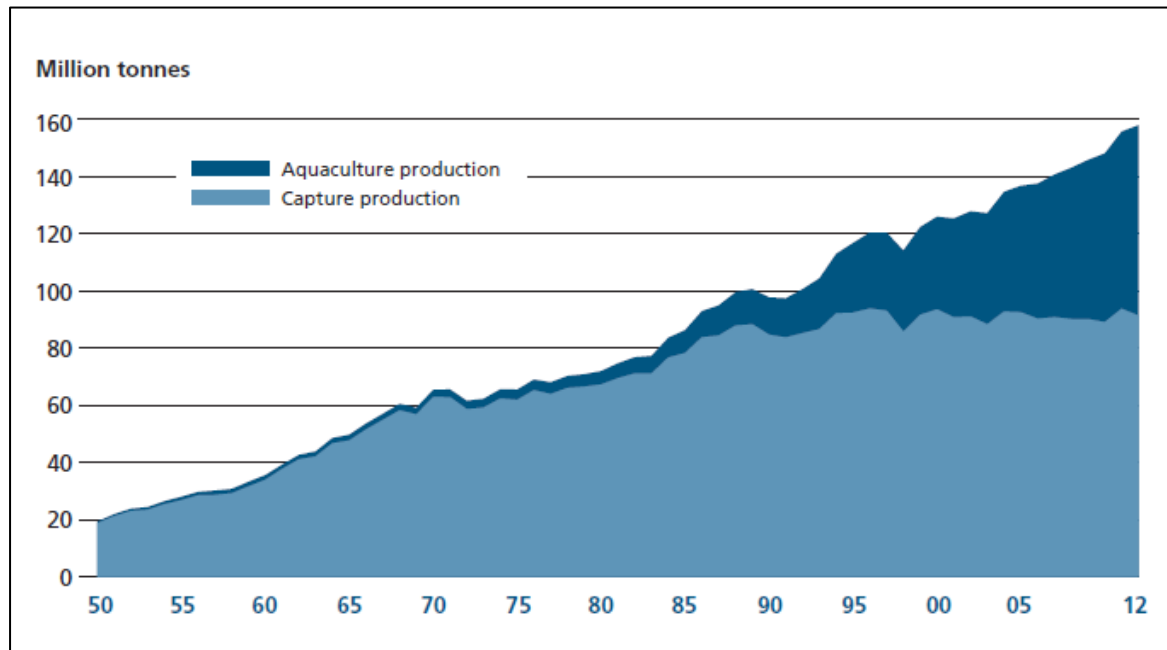
Figure 1.1 The status of world capture fisheries and aquaculture production from 1950 – 2012.

Source: FAO,(2014)

As a result of general increased awareness of the importance of PUFAs, there has been a growth in fresh fish consumption within both developed and developing societies over the past decades (Kearney, 2010). Developed societies need to decrease the intake of n-6 fatty acids (AA) and increase their n-3 fatty acid consumption (EPA and DHA) (Simopoulos, 2000). However, greater challenges exist in developing societies, with the most important objective being the need to meet the nutritional gap of people suffering from dietary deficiencies, supplied in an acceptable manner, so as not to try and change dietary habits (Li and Hu, 2009).

The Food and Agriculture Organization of the United Nations (FAO), (1988) introduced a definition of aquaculture which reduces its confusion with capture fisheries: "Aquaculture is

the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stocks being cultivated. For statistical purposes, aquatic organisms which are harvested by an individual or corporate body which has owned them throughout their rearing period contribute to aquaculture, while aquatic organisms which are exploitable by the public as a common property resources, with or without appropriate licences, are the harvest of fisheries".

World aquaculture production has grown steadily in the last five decades (see Figure 1.1), increasing dramatically over the past 20 years to become the fastest growing animal food production sector in the world, which in turn has led to job provision for millions and supported the livelihoods of hundreds of millions, whilst aiding food security and helping to meet the financial needs of poor households in developing countries (Thurstan and Roberts, 2014). Finfish dominate global aquaculture production by tonnage, categorized into inland aquaculture and mariculture. Inland aquaculture generally uses freshwater, but some production operations use saline water in inland areas and inland saline-alkaline water (such as in China) (Zhao *et al.*, 2010). Mariculture includes production operations in the sea and intertidal zones as well as those operating with land-based (onshore) production facilities and structures (FAO, 2014).

Asia accounts for approximately 88 percent of world aquaculture production by volume (FAO, 2014), with China responsible for most of the growth in finfish availability, owing to the dramatic expansion in its fish production systems for commercial exports, coupled with freshwater farming being a relatively easy entry point for practicing aquaculture for small scale producers when compared with mariculture (FAO, 2014). World aquaculture

production in 2012 comprised of 44.2 million metric tonnes (mmt) of finfish, of which 38.6 mmt was from freshwater finfish (inland aquaculture) and 5.6 mmt from mariculture. Mollusc production accounted for 15.2 mmt (including shell weight), crustacea 6.4 mmt and other aquatic species (e.g. sea cumbers, sea urchin, etc.) 0.9 mmt. (FAO, 2014), as shown in Figure 1.2.
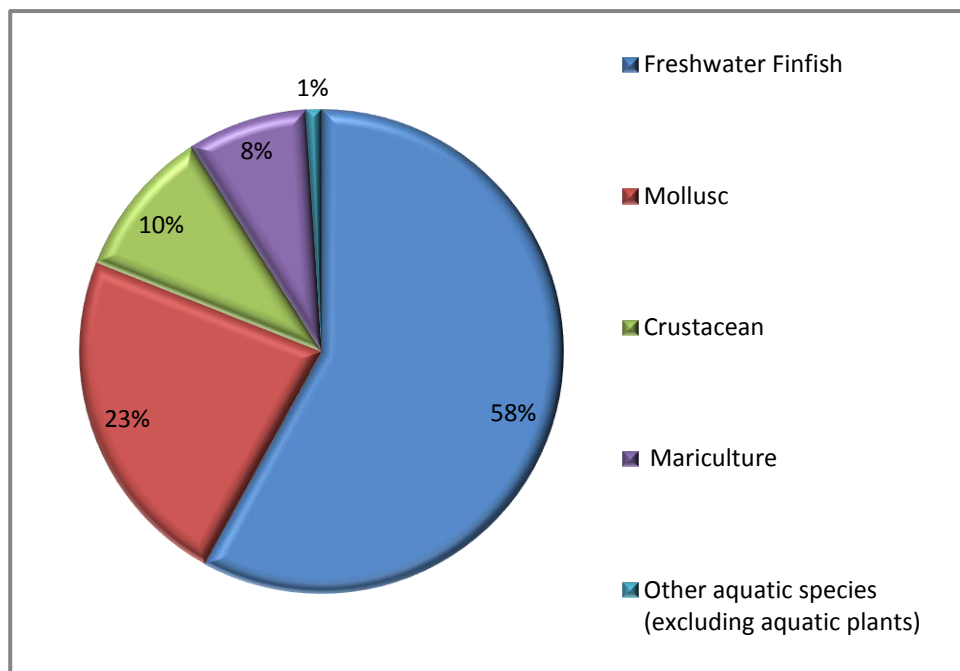
Figure 1.2 Percentage distribution of global aquaculture production in 2012 (excluding aquatic plants). Source: Adapted from FAO (2014) data.

Global freshwater aquaculture is primarily dominated by production of finfish e.g. Cyprinid (carp) and Cichlid (tilapia), most of which are herbivorous species and to a greater extent filter feeders or non-fed fish that are low in the food chain (FOA 2012). Their production, in theory, requires very little or no fishmeal (FM) and fish oil (FO) feed inputs enabling a great potential for expansion in the future (FAO, 2014). Freshwater fish farming has made the greatest direct contribution to the supply of affordable protein supplies from non-fed fish

species that are excellent providers of nutrients and are highly acceptable in many food cultures and do not necessarily compete for already limited feed resources, particularly for people still in poverty living in developing countries such as in Asia, Africa and Latin America (FAO, 2012).

A profitable and environmentally responsible aquaculture production system requires efficient management of feed resources. These feed resources provide fish with all the appropriate nutrients in the form of protein, fat, carbohydrate, vitamins and minerals. The accelerated development of the aquaculture industry worldwide has instigated initiatives to source alternative dietary ingredients. The vast majority of aquaculture producers use feed conversion ratios (FCRs) as an index for the efficiency of feed usage (Boyd, 2005). FCRs can vary due to a number of factors, including species, feed type and quality, production system, feeding technique, and water quality conditions (Boyd, 2005)(See Fig. 1.3).
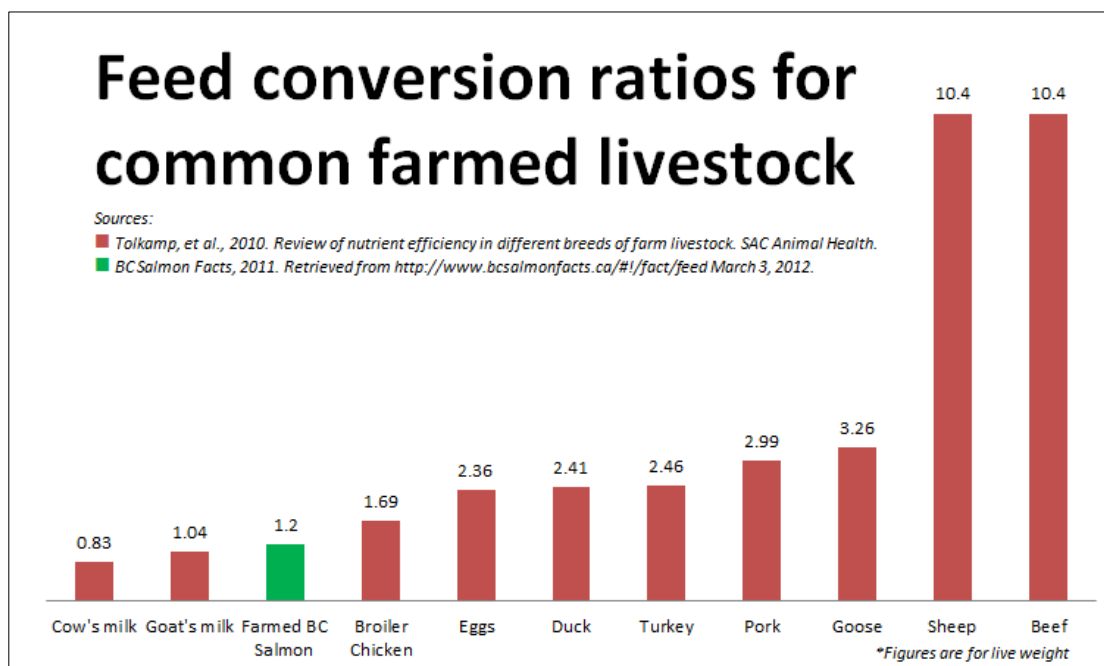
Figure 1.3 Feed Conversion Ratio (FCR) of feed mass input to body mass output in various production species. Source: (FAO, 2011).

Fish are more efficient at converting protein than most common farmed terrestrial livestock (see Fig 1.3), as fish are poikilothermic and do not require energy inputs to maintain an internal body temperature, whereas terrestrial animals are homoeothermic and must use energy to maintain internal body temperature (Bowyer *et al.*, 2013). Also, fish expend less energy to maintain position in the water column when compared to terrestrial animals that must contend with gravity (Skretting, 2016). The ratio of wild caught fisheries input to fishmeal fed farmed fish output has fallen to approximately 0.7 for the aquaculture sector as a whole (excluding filter feeders) (Naylor *et al.*, 2009) and this has been achieved by a better understanding of individual species dietary requirements (Dinh Van *et al.*, 2011) as different species vary considerably in the manner in which FM and FO affect their FCRs. Generally superior feed conversion can be achieved by high quality FM and FO for carnivorous fish species (Drew *et al.*, 2007). High quality fishmeal provides balanced amounts of all essential amino acids and especially PUFA - containing phospholipids (DHA and EPA) needed for optimum development, growth and reproduction (Hardy and Tacon, 2002), challenges lie in finding alternative sources.

1.2 Aquafeed- supply issues & alternatives

1.2.1 Fish meal & Fish oil

As the human population increasingly appreciates seafood as a key source of healthy animal protein and Omega 3 fatty acids, aquaculture activities are more dependent on an increased share of the world's wild fish resources for the production of fish meal and fish oil. This increase is leading to concern that the rapid expansion of the aquaculture industry may be constrained in the future by dependence on low-value, marine fish, unless alternatives are investigated (Hardy and Tacon, 2002; Tacon *et al.*, 2010). During the first half of the 1990s, salmon (*Salmo and Oncorhynchus* spp.) and shrimp (*Litopenaeus* spp.) aquaculture were two of the most intensive users of FM, because of its low cost, high protein content (65-72% crude protein), well-balanced proportion of all ten indispensable amino acids that meet the requirements of fish species, high digestibility and the fact that it is a source of essential fatty acids (EPA & DHA). This led to criticism of the salmon farming industry for being a net consumer of marine resources, in the form of FM and FO used in its feeds (Naylor *et al.*, 2009). Since 2000, the price of global FM & FO has increased from ca. US$450 per metric ton to almost US$2000 per metric ton in July 2014 (Tacon and Metian, 2008; Indexmundi, 2014).

Unappetizing wild caught, small bony pelagic fish are predominantly used in the production of FM. FM is a brown flour obtained by the process of heating and cooking the fish using steam to rupture the fat cells, pressing the cooked fish to separate as much of the liquid fraction from the solid fraction, drying and finally milling to a required particle sizes. The liquid fraction is further refined through a series of screens and centrifuges to remove any residual solids, then to further separate the water from the oil (FAO, 1986). The top global

producers of FM from this process are Peru and Chile (FAO, 2015), with production from species such as jack mackerel (*Trachurus symmetricus*), anchovy (*Engraulidae* sp.), and sardines (*Clupeidae* sp.).

Production of both fishmeal and fish oil from Peru/Chile in the first quarter of 2015 was very low due to the cancellation of the second anchovy fishing quota in 2014. Prices have gradually settled down from their peak witnessed at the end of 2014. The projection of a strong El Niño phenomenon has caused Peruvian fishing companies to expedite their fishing activities before fish are increasingly driven to the south or deeper water (FAO, 2015).

The price of FM and FO is highly dependent upon global factors such as the ongoing issues of climate change, the cyclic phenomenon of El Niño (Oki and Kanae, 2006) and rare natural disasters like the Chilean earthquakes of 1995, 2005 and 2010, all causing production in Peru and Chile to be substantially reduced; the subsequent change in the quality and quantity of FM greatly affected global supplies and prices (Tveteras and Tveteras, 2010). The limited availability of FM at these times has led to some concerted efforts to replace FM, analysing possible physiological or metabolic consequences (Kaushik and Seiliez, 2010), as well as the economic and environmental implications.

Despite efforts to substitute FM & FO in aqua feeds, many consumers, producers, purchasers and policymakers remain unclear about the suitability and sustainability of alternatives (Naylor *et al.*, 2009). To be a viable alternative for FM or FO, a potential ingredient must possess certain characteristics. These include nutritional suitability, ready availability, as well as ease of handling, shipping, storage and use in feed production. In addition, feeds are selected on the basis of fish health and performance, consumer acceptance, minimal pollution, ecosystem stress, and human health benefits (Naylor *et al.*, 2009). However, the primary driver is competitive pricing, as this is essential for the

adoption of a non-fish alternative to be utilised in feeds for aquaculture enabling expansion and growth (Naylor *et al.*, 2009). General dietary protein levels required for many of the commercially farmed species are known to average 40-50% for marine shrimp, 28-32% for catfish, 32-38% for tilapia and 38-42% for hybrid striped bass (Helfrich, 2009). Protein requirements are usually lower for herbivorous and omnivorous fish than they are for carnivorous fish, and are higher for larval and juvenile stages than that at the on-growing stages (Cho and Kim, 2011).

As the costs of FM continue to increase and fluctuate across global markets there has been an increased shift towards the substitution of FM with good quality protein sources by amalgamating animal by-product meals, fish by-products, plant products or as individual components inclusion in complex formulated diets that are species specific.

Andrew Mallison, Director General of International Fishmeal & Fish Oil Organization (IFFO) recently estimated that >35% of the world's fishmeal could come from by-products in the future, such as fish trimmings and discards, stating that "there is an environmental imperative to process by-products rather than dumping them at sea or on land "(IFFO, 2013).

1.2.2 Animal by-product meals

Animal by-products (ABP) are defined in Article 3 of Regulation (EC) No 1069/2009 as "entire bodies or parts of animals, products of animal origin or other products obtained from animals that are not intended for human consumption" and are divided into 3 categories. Category 1 ABPs are classified as high risk, includes carcasses and all body parts of animals suspected of being infected with TSE (Transmissible Spongiform Encephalopathy), which were quickly prohibited in 2000 for use in European Union "feedingstuffs" after the outbreak of Bovine Spongiform Encephalopathy (BSE), commonly known as mad cow disease (Fries, 2003). Category 2 is also classified as high risk and includes fallen stock/ dead animals (all species) and animals rejected from abattoirs due to having infectious diseases. Category 3 ABPs are classed as low risk. They include carcasses or body parts passed fit for humans to eat, at a slaughterhouse, products or foods of animal origin originally meant for human consumption but withdrawn for commercial reasons, not because it is unfit to eat, shells from shellfish with soft tissue, aquatic animals, aquatic and terrestrial invertebrates, hides and skins from slaughterhouses, animal hides, skins, hooves, feathers, wool, horns, and hair that had no signs of infectious disease at death.

During the slaughter and processing of farmed livestock, approximately 33 to 43% by weight of the live animal is removed and discarded as an inedible waste material (FAO, 2002a). Materials such as fat trim, meat, viscera, bone, blood and feathers are collected and processed by the rendering industry to produce high quality fats and proteins products such as meat and bone meal, blood meal, poultry by-product meal (poultry meal) and feather meal are all termed as processed animal proteins (PAPs), all of these have been traditionally used in the animal feed industry (with the exception of ruminant blood meal).

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These animal products encompass a well-balanced amino acid profiles, complementing all of the essential amino acids required for favourable fish health and growth (including lysine and methionine, which are generally deficient in most plant based ingredients (Nunes *et al.*, 2014). Although poultry meat meal and feather meal PAPs are permitted by the EU for inclusion in aquafeeds, there is still much resistance from the retail industry due to public perception of their safety (Nunes *et al.*, 2014).

1.2.3 Fish by-products

Fish trimmings and carcass are commonly called by-products and if treated correctly, classified as category 3 by-products according to EU regulation, meaning parts of animals that are fit for, but not intended for human consumption (EC No 1774/2002). Recent estimates suggest that >35% of fishmeal production is from by-products obtained from processing of fish (Olsen *et al.*, 2014).

Annually large amounts of underutilized by-products are generated from global fisheries as by-catch / discard that have the potential to make a valuable short- to medium-term contribution to the global supply of FM and FO as prices continue to erratically fluctuate (FAO, 2012). Furthermore, the European Commission has adopted a series of discard plans that came into operation on 1 January 2015. This marks the first phase of the landing obligation which will be introduced gradually between 2015 and 2019 for all commercial fisheries, that will first apply to pelagic species including blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*), herring (*Clupea harengus*), horse mackerel (*Trachurus trachurus*), mackerel (*Scomber scombrus*), sand eel (*Ammodytes tobianus*), sprat (*Sprattus sprattus*) and any quota fish which are below the minimum landing size (DEFRA, 2014). Whilst commercial outlets dealing with by-products and the utilization of discards foresee

an opportunity to expand their businesses, it may be possible that this legislation is going to act as a disincentive for most fishermen due to the low prices paid by the fish by-product processing companies in comparison to the potential revenue from supplying for human consumption (Mangi and Catchpole, 2014).

1.2.4 Plant protein

The most attractive alternative, and therefore most widely utilised, FM substitutes are plant-derived nutrient sources such as soybean meal, rapeseed (canola) meal, sunflower seed meal, lupin seed meal, cottonseed meal, pea meal, wheat and corn gluten to name a few (Medale *et al.*, 2013). The comparable protein percentages of animal proteins, plant protein and fish meal used in aquafeeds and the price per metric ton globally are listed in Table 1.1.

Table 1.1: Global prices of ingredients used in aquafeeds (US$/tonne).
Adapted from index mundi directory 2015 (http://www.indexmundi.com/) and table adapted from Hardy and Tacon, FAO (2002). Prices correct as of 01/06/15.

Ingredient	Percentage Protein	Price per mt ($)
Anchovy meal (FM)	60-72	600 - 1000
Brewer's yeast	54	800 - 1200
Corn gluten	60	750 - 800
Wheat gluten	70-80	500 - 550
Soybean meal	48	400 - 600
Rapeseed meal	61	300 - 600
Meat and Bone meal	51	400 - 500
Blood meal	89	400 - 500
Poultry By-product meal	60	350 - 600
Feather meal	83	350 - 450

When compared to fish meal, plant proteins exhibit several limitations. Palatability, digestibility and cost per unit of protein, accompanied with unbalanced amino acid composition are factors that need consideration when they are used in formulations. Further problematic challenges are associated with the use of plant proteins in diets for finfish are linked to the presence of anti-nutritional factors (ANFs), the chemical defence mechanisms against predators which are located within plants (Ferrando, 1983), some of which have been widely documented to have an adverse effect on the digestive function of fish (Vandeningh *et al.*, 1991; Vandeningh *et al.*, 1996).

The ANFs listed in Table 1.2, (see overleaf) can be divided into two categories: heat-labile and heat-stable ANFs. Heat-labile ANFs are destroyed or altered by heat and include trypsin inhibitors, phytates, lectins, and anti-vitamins, whilst heat-stable ANFs include carbohydrate or soluble fibre, saponins and allergens (Greathead, 2003).

Heat labile ANFs should be destroyed during processing, or they could harm certain fish species by causing disorders, such as gut enteritis, particularly in carnivorous species (Fortes-Silva *et al.*, 2011). The presence of certain ANFs in plant based diets is often not lethal, but may impinge upon fish welfare, this may be due to factors such as non-available Phosphorus, insoluble protein and vitamins, low diet digestibility and poor palatability, increasing the susceptibility to secondary disorders and infectious diseases (Francis *et al.*, 2001). Little information is available in the literature concerning the effects of specific ANFs on specific fish species or the levels of different ANFs in aquafeeds containing plant ingredients (Carter, 2003; Raes *et al.*, 2014); further understanding into the nutritional, physiological and ecological effects of ANFs on fish species is needed, through studies using

purified individual anti-nutrients and their mixtures in proportions similar to those in alternative nutritional sources in fish feeds.

Table 1.2: Important anti-nutritional factors present in some commonly used alternative fish feed ingredients.
Adapted from Francis *et al.,*(2001)

Alternative Feed Ingredients	Anti-nutritional factors
Soybean meal	Trypsin inhibitors, glucosinolates, phytic acid, saponins, anti-vitamins, phytohaemagglutinins
Broad/Faba beans	Protease inhibitors, phytic acid, saponins, tannins, Dihydroxyphenylalanine
Rapeseed meal	Protease inhibitors, glucosinolates, phytic acid, tannins, erucic acid
Lupin seed meal	Protease inhibitors, saponins, phytoestrogens, alkaloid, estrogenic factors
Pea seed meal	Protease inhibitors, cyanogens, phytic acid, saponins, anti-vitamins

An FAO (2002b) report identified some major constraints with using alternative plant based protein sources, such as erratic quality, generally lower digestibility, lower availability of some essential amino acids, palatability problems, and in some cases the presence of anti-nutritional factors, as well as limited availability due to a possible human consumer conflict as a direct protein source, all of which have limited the replacement of fishmeal by plant proteins. However the addition of exogenous enzymes, such as trypsin, amylase and pepsin amongst others provide additional powerful tools that can inactivate some ANFs and enhance the nutritional value of plant-based protein in feeds (Classen, 1996), providing a natural way to transform complex feed components into bioavailable nutrients. The

addition of enzymes to feed can improve nutrient utilization, reducing feed cost and the excretion of nutrients into the environment (Kumar *et al.*, 2012; Castillo and Gatlin, 2015).

1.2.5 Krill

Krill are a family of pelagic marine crustaceans, *Euphausiids*, which are widespread throughout all the oceans, comprising of over 80 species, most of which are planktonic (Nicol *et al.*, 2012). Recent developments in harvesting technology and significant growth in the market for krill- derived products indicate renewed activity in exploiting this resource, with the species *Euphausia superba* (Antarctic krill) currently receiving the vast majority of commercial interest for aquaculture, pharmaceutical and medical products (Nicol *et al.*, 2012). Krill meal is a comprehensive source of protein that contains all the essential amino acids required for fish health and growth (Han-Soo *et al.*, 2014). Furthermore, krill oil is an excellent source of EPA and DHA as well as carotenoids in the form of astaxanthin. In addition, unlike most other fish oils, the major part of EPA and DHA in krill oil occurs naturally in phospholipid and not in the triglyceride form (Ali-Nehari *et al.*, 2012).

However, there are two natural components of krill, chitin (possibly reducing digestibility of aquafeeds) and fluoride (considered a bio-accumulative toxin), that pose potential obstacles to their use in aqua feeds. Processing costs are also high and therefore krill is likely to remain a high-value additive to aquaculture feeds rather than the primary ingredient (Nicol and Endo, 1999).

1.2.6 Single cell proteins (SCP)

The term single cell protein (SCP), coined in 1966 by Carroll L. Wilson (Goldberg, 1985), refers to dried microbial cells and means to embrace microbial biomass products produced by fermentation that are used as a feed ingredient for livestock. SCPs possess many desirable characteristics, such as high protein levels (on average 45 – 50%, although some strains of bacteria can contain approximately 80%) and the presence of important lipids, vitamins and minerals (Anupama and Ravindra, 2000). Due to these characteristics, substantial research has been performed over the years, dating back to early studies carried out in the 1970's (Windell *et al.*, 1974; Sanchezmuniz *et al.*, 1978). These and many other studies have involved yeast- (Moerschbaecher *et al.*, 2014) ,fungal- (Belal, 2008), algal- (including live unicellular algae) and bacterial SCPs (Wang and Levin, 2009). Whether derived from plant or animal by-product wastes or independently through autotrophic growth, they have been explored in experimental diets for many important aquaculture species, such as rainbow trout (*Oncorhynchus mykiss*) (Dallaire *et al.*, 2007), Koi carp (*Cyprinus carpio*) (Korkmaz and Cakirogullari, 2011), Nile tilapia (*Oreochromis niloticus*) (Bob-Manuel and Alfred-Ockiya, 2011; Bob-Manuel and Erondu, 2011), channel catfish (*Ictalurus punctatus*) (Li *et al.*, 2009) and Atlantic cod (*Gadus morhua*) (Walker and Berlinsky, 2011) with varying degrees of success. The positive characteristics of SCPs generally include short generation times and rapid growth rates (Anupama and Ravindra, 2000). In addition they are capable of growing on a huge variety of raw materials (Table 1.3, below), requiring limited land surface area for production (Ratledge, 1975).

Table 1.3 Micro-organisms and substrates used for single cell protein production.

Source: Bhalla *et al*,(2007)

Microorganism	Substrate
Bacteria	
Aeromonas hydrophila	Lactose
Bacillus megaterium	Non-protein nitrogenous compounds
Lactobacillus sp*.*	Glucose, amylose, maltose
Methylophilus methylotrophus	Methanol
Fungi	
Aspergillus fumigatus	Maltose, glucose
Penicillium cyclopium	Glucose, lactose
Rhizopus chinensis	Glucose, maltose
Yeast	
Amoco torula	Ethanol
Candida novellas	n-Alkanes (Hydrocarbons)
Saccharomyces cerevisiae	Lactose, pentose, maltose
Algae	
Chlorella sp.	Carbon dioxide through photosynthesis
Spirulina sp*.*	Carbon dioxide through photosynthesis

1.3 Algae

1.3.1 The algal cell

Algae are aquatic organisms, lacking true roots, stems and leaves (Rogers, 2011). These predominantly photosynthetic organisms exhibit a variety of forms, from single-celled phytoplankton (microalgae) suspended in the water column, to large multi-cellular seaweeds (macroalgae) attached to the ocean floor via holdfasts (Rogers, 2011).

Microalgae have chlorophyll *a* as their primary photosynthetic pigment (Yaakob *et al.*, 2014); this has enabled them to evolve into a large and diverse group of unicellular autotrophic and heterotrophic organisms which can be exploited to produce a wide range of metabolites such as proteins, lipids, carbohydrates, carotenoids and vitamins (Koller *et al.*, 2014) (see Table 1.4, overleaf). Algae are at the very base of the aquatic food chain and have been cultivated as a food source for humans dating back more than 1,600 years (317 to 420 A.D.) to the time of the Eastern Jin Dynasty when the Chinese used *Nostoc* spp. (cyanobacteria) to survive during famines (Potts, 1997).

In their simplest form, microalgae were the first photosynthetic inhabitants of Earth, splitting carbon dioxide and water molecules, and exploiting solar energy in the form of photons, in order to manufacture soluble sugars (glucose) as an instant energy source, producing oxygen as an extremely important by-product, thus giving rise to the atmosphere which almost all living organisms depend upon today (Schirrmeister *et al.*, 2015). Algae structures are primarily geared towards energy conversion with simple development that allows them to adapt to prevailing environmental conditions and prosper in the long term (Brennan and Owende, 2010).

Table 1.4 Micro-algae cellular metabolites and their attributes in the context of aquaculture nutrition.

Metabolite	Attribute	Reference
Essential Amino acids (EAA) (Protein)	Essential in a well-balanced mixture for all protein synthesis. Main constituent of the fish body, thus sufficient dietary supply is need for optimum growth	(Dabrowski *et al.*, 2010a; Finn and Fyhn, 2010)
Essential Fatty Acids (EFA) (Lipid)	Important source of metabolic energy enabling dietary lipids to be used for energy metabolism.	(Tocher, 2010)
	In the form of Poly-unsaturated fatty acids (PUFA) which can deliver valuable immune and cardiovascular promoting attributes on to the fish and subsequently the consumer	(Sargent *et al.*, 1995; Arthur, 2009; Shapira *et al.*, 2009)
Carotenoids	Pigment composition is characterized by chlorophyll *a* (not chlorophyll *b* or chlorophyll *c*), β-carotene, as major pigments with zeaxanthin, canthaxanthin and astaxanthin, as minor ketonic groups.	(Lubian *et al.*, 2000)
Anti-oxidants sp.	With respect to their ability to reduce the incidence of some chronic diseases where free radicals are involved	(Christaki *et al.*, 2013)
Vitamins	Hold a unique role in maintaining health and growth	(Brown *et al.*, 1999)

1.3.1.1 Algal protein

Comparison of data for protein content in algae is very difficult, primarily due to differences in the analytical methods employed, additionally most analyses of algal proteins are based on estimations of "crude protein", obtained by hydrolysis of the algal biomass and estimated as "total nitrogen", commonly used in evaluating food and feed stuffs (Barbarino and Lourenco, 2005). Proteins are composed of different amino acids and hence the nutritional quality of a protein is determined basically by the content, proportion and availability of its amino acids (Becker, 2007a). Approximately two hundred amino acids occur in nature, but only about 20 amino acids are considered common. Although animals do not have a specific protein requirement *per se*, 10 amino acids are termed "essential" and cannot be synthesized: methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine. Among all the essential amino acids, lysine and methionine are the first limiting amino acids (Santiago and Lovell, 1988). Many micro-algal species contain relatively high amounts of lysine, but some are deficient in the sulphur-containing amino acids, cystine and methionine (Becker, 1983).

It has been suggested that carnivorous fish, like Atlantic Salmon, are not particularly efficient in digesting particle nutrients from algae (Norambuena *et al.*, 2015) and feeding algae to rainbow trout, another carnivorous fish, can be detrimental for growth (Barrows and Frost, 2014). Furthermore, previous studies suggest that fish are not able to digest more than 45–56% protein from algae (El-Sayed, 1999), not because of protein quality, but because of the limited ability in the hydrolysis of complex polysaccharides present in algae (Montgomery and Gerking, 1980).

The inclusion level of microalgae meals in fish diets may be limited due to their protein content and other factors such as specific nutrient requirements in different fish species,

and ingredient digestibility. For example, despite the high protein content in Spirulina meal, it could only replace up to 50% of fish meal in diets for silver sea bream and was suitable at even lower replacement levels for other species (Elsayed, 1994).

1.3.1.2 Algal lipid

Long chain polyunsaturated *n*-3 fatty acids (LC-PUFAs) are of increasing interest, due to their many positive effects for human health (Laidlaw *et al.*, 2014) and their use in aquaculture (Ryckebosch *et al.*, 2014). Before now, seafood was the main source of *n*-3 LC-PUFAs (Martins *et al.*, 2013), however alternative sustainable sources for *n*-3 LC-PUFAs are being developed (Miller *et al.*, 2008). Vertebrates, including fish cannot produce PUFAs *de novo*; instead, these substances are transferred through the trophic chain to organisms of higher levels, with microalgae as the primary producers (Sargent, 1997). Therefore, it is unsurprising that marine microalgae have been targeted as potential candidates for industrial production of *n*-3 LC-PUFAs (Srigley and Rader, 2014) such as eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) (Behrens and Kyle, 1996; Vazhappilly and Chen, 1998; Adarme-Vega *et al.*, 2014; Ryckebosch *et al.*, 2014; Meng *et al.*, 2015).

Contained within the chloroplast is the thylakoid membrane which contains pigments to capture differing wavelengths of light energy (Lodish *et al.*, 2000). The most important pigments are chlorophylls (olive green in colour) and accessory pigments known as carotenoids (usually yellow or orange)(Bartley and Scolnik, 1995). The light reaction, also known as photolysis reaction, splits water into oxygen, electrons, and protons in the first stage (the light-dependent reaction), to convert into chemical energy contained

as Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) and Adenosine Triphosphate (ATP)(Lodish *et al.*, 2000).
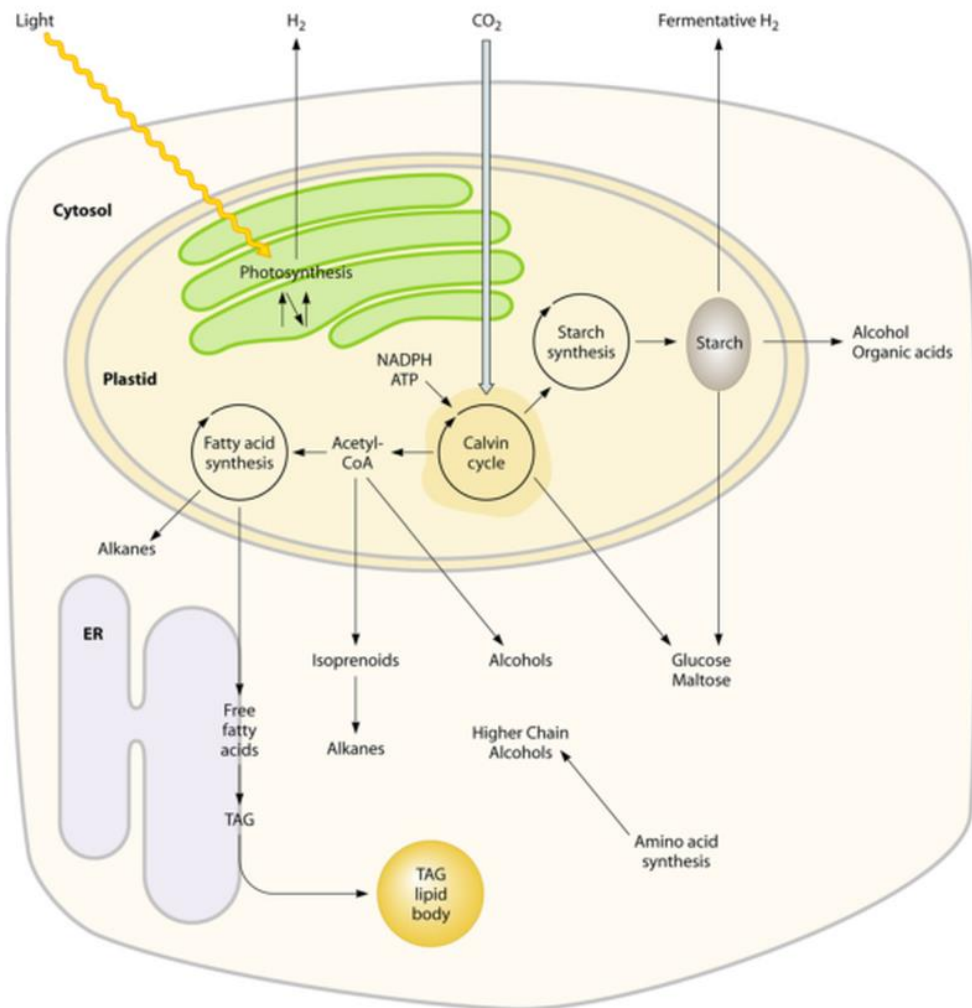
Figure 1.4 Metabolic pathways and processes involved in energy and lipid production in a microalgal cell.
ER = Endoplasmic Reticulum. Source: Radakovits *et al.*, (2010)

During the second stage, called the light-independent reaction (formerly known as the dark reaction), the Calvin-Benson cycle uses the ATP as an energy source and consumes NADPH as a reducing molecule that provides hydrogen atoms that help to form glucose (see Figure 1.5, previous page)(Radakovits *et al.*, 2010). These are the necessary energy carriers that are substrates for the production of an intermediary chemical compound, glyceraldehyde-3-phosphate (G3P), which can enter either the starch or monosaccharide biosynthesis pathways depending on conditions within the cell.

Fatty acids are the building blocks for triacylglycerols (TAGs) and all other cellular lipids (Rezanka *et al.*, 2014), Fatty acids, the building blocks for TAGs and all other cellular lipids, are synthesized in the chloroplast using a single set of enzymes, of which acetyl CoA carboxylase is the universal carbon donor, used for the vital role of fatty acid biosynthesis (Hu *et al.*, 2008). It is supplied via multiple pathways from various origins and in any step, a synthesized carbon chain can be metabolized into various products including free fatty acids, TAGs and phospholipids (Hu *et al.*, 2008). However, the expression of genes involved in fatty acid synthesis is poorly understood in microalgae (Cooksey, 2015).

1.3.1.3 Algal carotenoids & antioxidants

For photosynthesis, both carotenoids and chlorophylls are bound to peptides to form pigment-protein complexes in the thylakoid membrane within the microalga cell (Green and Durnford, 1996). Carotenoids cannot be synthesized by species in the animal kingdom, but are essential as building blocks for cellular membranes, with animals obtaining carotenoids from their diets (Lorenz and Cysewski, 2000). Carotenoids are divided into two classes: xanthophylls, which contain oxygen, and carotenes, which do not (Stahl and Sies, 2003).

Carotenoids serve two key roles in plants and algae: they absorb light energy for use in photosynthesis and they also protect chlorophyll from photo-protection. Carotene compounds are arranged as two small six-carbon rings connected by a "chain" of carbon atoms (Britton, 1995). As a result, they do not dissolve in water and must be attached to membranes within the cell (Subczynski and Wisniewska, 2000).

Carotenoids are known to be important in human health as antioxidants (Rao and Rao, 2007). There is also evidence that they are involved in other biological functions, such as anti-inflammatory and anti-tumoral features (Guedes *et al.*, 2011) and there are also reports that carotenoids can protect human skin against UV-induced damage (Ahmed *et al.*, 2014), working as efficient free-radical scavengers. The conclusion that major public health benefits could be achieved by increasing consumption of carotenoid-rich fruits and vegetables still appears to stand (Mayne, 1996) and data originating from epidemiological studies and clinical trials strongly support the observation that adequate carotenoid supplementation from fruit, vegetable and algal sources may significantly reduce the risk of several disorders (Fiedor and Burda, 2014). However, more research is required in this area.

Carotenoids are used in aquaculture feeds to provide the colour associated with consumer products, such as the bright vibrant colours of ornamental fish (Sun *et al.*, 2012). The same carotenoid, astaxanthin, found in wild salmon is used in aquafeeds to impart a natural, pink-red colour to farmed salmon fillets (Torrissen, 1995). With regards to consumer preference it is the colour that matters, whether this is derived from pigmentation deposition within flesh or skin.

1.3.2 Microalgae potential

The search for alternative protein sources and renewable energy has made the potential uses of microalgae a hot topic within the field of sustainable development (Chu and Majumdar, 2012). Recently, there have been considerable advances in algal biotechnology (Bechet *et al.*, 2015). Micro-algal (phytoplankton) cultivation has been researched for well over fifty years, but most efforts have been devoted to developing pharmaceutical and nutraceutical products (Shukla and Dhar, 2013). It is estimated that possibly as many as several million species of algae exist (Norton *et al.*, 1996), but only a fraction of these (approximately 30,000) have been studied and analysed (Richmond, 2003). Mass cultivation of microalgae dates back to the mid-twentieth century, whether it is for bio-energy production (Alp and Cirak, 2012), water pollution remediation (Dubey *et al.*, 2011), CO_2 capture (Otsuki, 2001), landfill management (Spork and Kongeter, 1997) or as a food/ feed source (Medale and Kaushik, 2009; Christaki *et al.*, 2010; Christaki *et al.*, 2011), the potential for these photosynthetic microorganisms is just starting to be explored. With fewer than 30 micro-algal species in commercial production today, all have differing nutritional characteristics and properties with varying opportunities available (see Figure 1.6, overleaf). Fewer species still are cultivated as "living capsules of nutrition" for larval / early juvenile stages of fish / shrimp production and shellfish aquaculture alike. These continue to be either consumed as a direct source of nutrition in "green water techniques" (Skiftesvik *et al.*, 2003; Besbes *et al.*, 2010) or acquired indirectly from the gut of prey species such as rotifers (Lubzens *et al.*, 1989) and/or brine shrimp (*Artemia* sp.; (Sorgeloos *et al.*, 2001). Their continued use is due to their high level of digestibility, cell size range (2 to 30 µm, which meets the feed size requirement for numerous aquatic animals) and the available nutritional attributes that are generally accepted to enhance survival, as well as confer good growth

rates (Conceicao *et al.*, 2010). However, these organisms exhibit overall deficiencies in levels of polyunsaturated fatty acids (PUFA, e.g., DHA and EPA) that require enrichment to improve their nutritional quality before being fed to many marine fish larvae, essential for early growth stages (Reitan *et al.*, 1997; Garcia *et al.*, 2008; Kobayashi *et al.*, 2008). Several enrichment techniques have been developed, including microalgae, oil-based emulsions and microencapsulated preparations (Shields *et al.*, 1999).
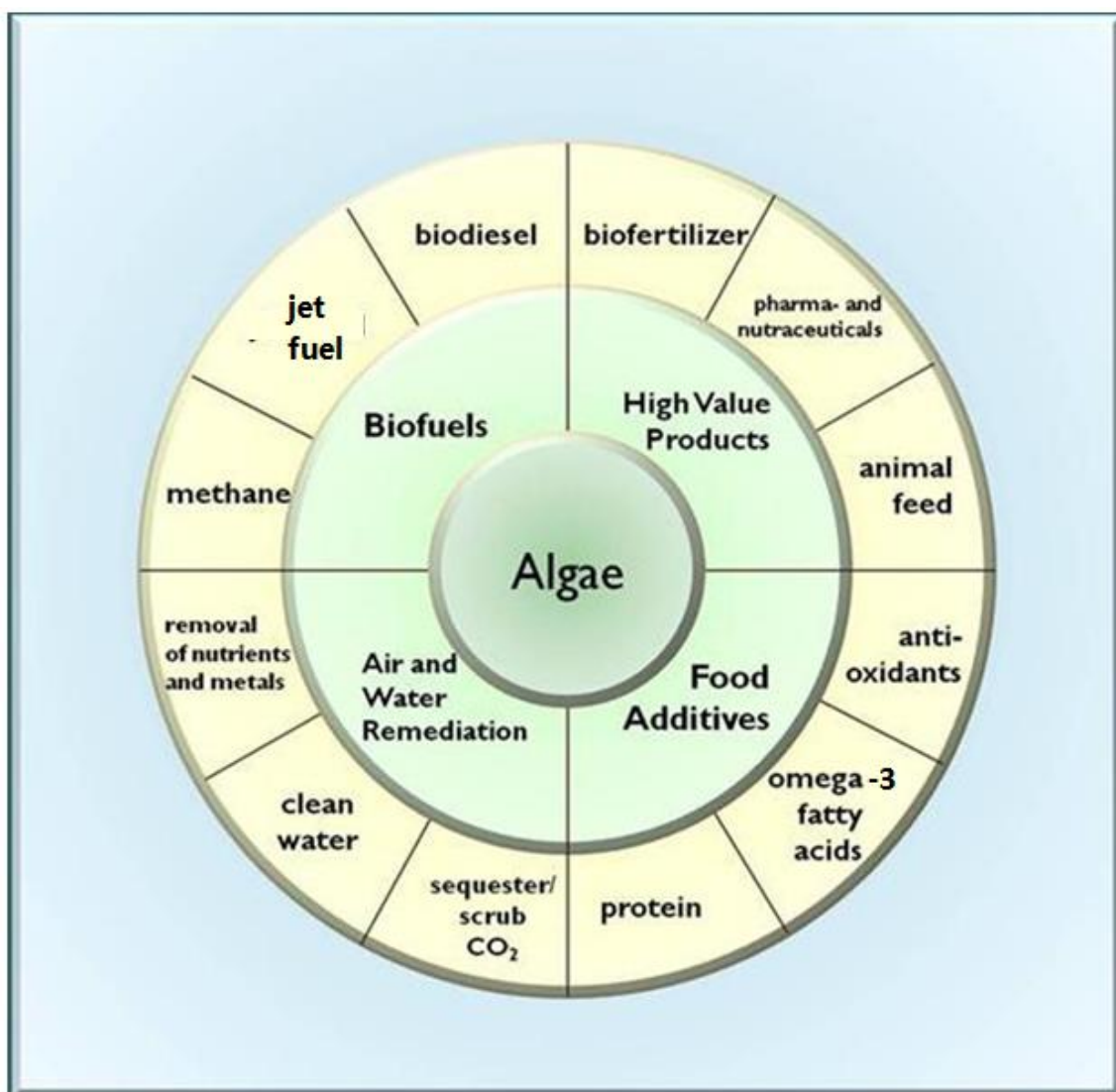
Figure 1.5 Varying opportunities for applications of commercial microalgae.

Source: http://www.dna2life.com/algae/5-astonishing-and-surprising-uses-algae

Furthermore, a study of note by Kokou *et al.* (2012) reported antibacterial activity of four microalgae species against six *Vibrio* species which resulted in absence or lower levels of all cultures studied. *Vibrio* spp. are common and potentially pathogenic bacteria present in marine and estuarine environments, living free or on the surfaces and in the intestinal contents of marine animals and are also found in freshwater habitats (Cabral, 2010). Further research is required in this area to explore possible additional antimicrobial potential.

Current research in microalgae is focused more on which strains have the capacity for creating high yield oil compounds (see Table 1.5) and which processes can extract these valuable products at a cost that makes them marketable on a competitive industrial level for the bio-fuel industry.

Table 1.5 Comparison of oil productivity of major crops and two microalgae.

Source: Scott *et al.,*(2010)

Crop/microalgae	Oil Content per ton (wt. % dry mass)	Oil Production (t/ha/y)	Biomass yield (L/ha/y)
Nannochloropsis spp.	Up to 50%	20-30	23,000 -34,000
Chlorella spp.	Up to 46%	7.2	8,200
Jatropha	30% (of seeds)	2.4	2,700
Oilseed rape (UK)	40-44%	1.4	1560
Soya	20% (of seeds)	0.48	544

It is well known that oils can be produced in significant amounts by some microalgal species. Energy production based on microalgae is often called a '3rd generation bio-fuel', as opposed to bio-fuels from edible (1st generation) and non-edible (2nd generation) plant parts (Ullah *et al.*, 2015). Economic and productivity analyses have revealed the high potential for

producing fuels from microalgae compared to current biodiesel of plant origin (Brennan and Owende, 2010).

Microalgae provide various potential advantages for bio-fuel production when compared with 'traditional' crops:

- Capability of producing oil all year round.

- Ability to grow on non-arable land, thus not affecting food supply or the use of soil and circumventing the issue of 'food versus fuel.

- Production of non-toxic and highly biodegradable bio-fuels.

- Microalgae need relatively less water than plant crops. Depending on the species, microalgae can grow in fresh water, brackish, saline, sea water or waste water and industrial effluents.

- Microalgae can utilize carbon dioxide; this provides greenhouse gas mitigation benefits.

- Microalgae can produce valuable co-products. For example, after oil extraction proteins can be used as animal feed, medicines or fertilizers, or fermented to produce ethanol or methane.

- However, disadvantages in the cost of de-watering and drying processes.

Source: Adapted from Gendy and El-Temtamy, (2013)

1.3.3 Technologies for microalgae biomass production

Micro-algal biomass is a renewable energy source and its importance will increase as the world is confronted with an energy crisis due to depletion of finite resources of fossil fuels (Ahmad *et al.*, 2011). Biomass energy (bio-energy) utilization has become of particular interest in recent years, with the most important biomass energy sources being wood and wood wastes, agricultural crops and their waste by-products, animal wastes, waste from food processing, and aquatic plants and microalgae (Demirbas, 2011). Microalgae, like other plant-based biomass resources, provide the mechanism for collection, conversion and storage of solar energy into chemical forms.

For algal biofuel production, the major factors presented as determining economic viability of production are productivity and harvesting costs. Currently, photoautotrophic production is the only method which is technically and economically feasible for large-scale production of algae biomass for human and animal consumption, although algae are not yet produced on a large enough scale to make a positive economic impact with regards to fuel production (Gerbens-Leenes *et al.*, 2014). Two systems that are currently used are (1) based on open system techniques and (2) closed "photobioreactor" (PBR) system technologies (Borowitzka, 1997); both offer pros and cons for microalgae production (see Table 1.6, overleaf). Photosynthetic efficiency is only relevant for autotrophic algae, but utilisation of sugars is more relevant for heterotrophically cultivated algae (Brennan and Owende, 2010), a process that is becoming increasingly utilized year-on-year (Ren *et al.*, 2013). Algae-based fuel technology offers a unique opportunity to produce both biodiesel and ethanol successively in a two-step process. This is possible as algae contain both oil (in algae cells) and sugars in the form of starch (the storage component) and cellulose (the cell wall component). After lipid extraction has occurred the resulting algal cake is rich in proteins, carbohydrates and

minerals (Ziolkowska and Simon, 2014). This algal meal is an available source of nutrients for use in animal and human nutrition (Spolaore *et al.*, 2006).

Table 1.6 An efficiency comparison of phototrophic and heterotrophic production systems for microalgae.

Adapted from Xu *et al.*, (2009)

	Open System	Closed System	Heterotrophic Cultivation
Contamination risk	High	Low	n/a
CO_2 losses	High	Low	n/a
Evaporation losses	High	Low	n/a
Light use efficiency	Poor	Excellent	n/a
Area/volume ratio	Low	High	High
Area required	High	Low	Low
Process control	Difficult	Easy	Easy
Biomass productivities	Low	High	High
Operational costs	Low	High	High
Investment costs	Low	High	Medium
Harvesting costs	High	Relatively low	Relatively low
Scale-up	Easy	Difficult	Easy

1.3.3.1 Open pond production systems

Open pond production systems are almost always located outdoors and rely on natural light for illumination. Although open pond techniques require less capital in start-up and overall equipment operation than alternative systems, production has long been associated with

potential contamination issues resulting in only a few species in production that can withstand highly alkaline or saline selective environments as a control method for invasive microbial contamination. Excessive space requirements due to high surface area requirements and limited location possibilities due to factors such as climate range are other challenges (Scott *et al.*, 2010). Ponds are usually a "raceway" design (see Figure 1.7), lined with plastic or cement, 15 - 30 cm deep, with motorized paddlewheels to provide a motive force to circulate nutrients and water around the race track, keeping algae suspended in the water column ensuring algae is exposed to sunlight for increased photosynthetic efficiency (Lee and Suh, 2003).
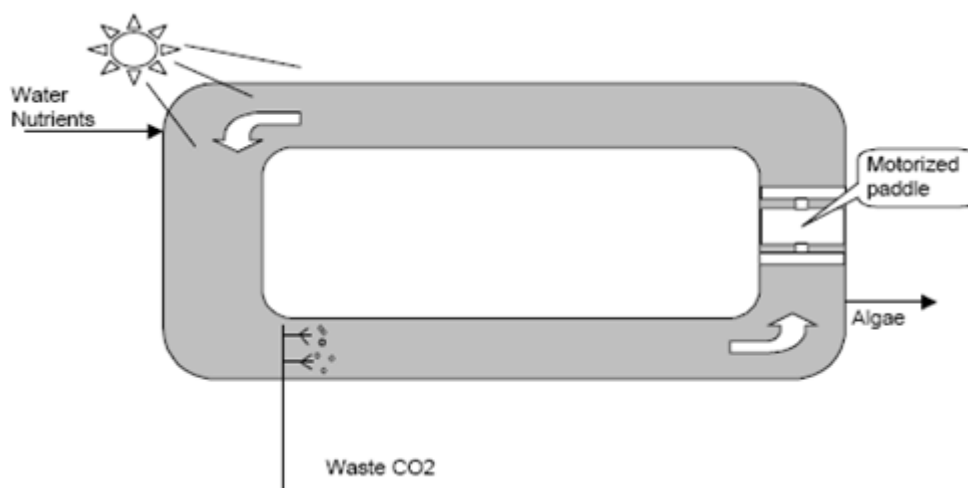
Figure 1.6 Open pond raceway production system.

 Source: http://www.nrel.gov/biomass/pdfs/24190.pdf

The ponds are kept shallow because of the need to keep the algae exposed to sunlight and the limited depth to which sunlight can penetrate the pond water limits self-shading (Pulz,

2001). The ponds are operated continuously, with water and nutrients being constantly fed to the pond, while algae-containing water is removed at the other end, with the system accumulating atmospheric carbon dioxide from the surroundings (Spolaore *et al.*, 2006). A harvesting system is required to recover the algae after this process, and this is discussed further in the next section. Although efforts have been made to improve open ponds with temperature control systems, supplies of appropriate nutrients, optimization of pond depth, carbon dioxide injection systems, etc., productivity remains fairly low compared to closed systems (Borowitzka, 1999; Radmann *et al.*, 2007). As a result of these limitations, focus has shifted to the development of cost-effective closed cultivation systems (Borowitzka, 1999).

1.3.3.2 Closed photobioreactor systems

Closed PBR systems consist of numerous designs- (e.g. tubular, flat-plated, column, etc.) that provide a high volume to surface area ratio that can support higher volumetric cell densities of photoautotrophs than an open pond production system. Regulation of carbon dioxide and dissolved oxygen levels in the bioreactor are key elements for efficient algal growth (Peng *et al.*, 2013). Lee and Bazin (1990) reported that tubular solar collectors with airlift systems are possibly the most promising for growing algae species (see Figure 1.8, overleaf). Circulating the culture by utilisation of an airlift device is especially attractive for several reasons: circulation is achieved without moving parts and this provides a robust culture system with a reduced potential for contamination (Li *et al.*, 2015) and the cell damage associated with mechanical pumping is avoided (Pirouzi *et al.*, 2014). This design also allows for oxygen exchange (to avoid an excessive build-up that inhibits photosynthesis) and the addition of carbon dioxide into the liquid culture medium (Xu *et al.*, 2009).
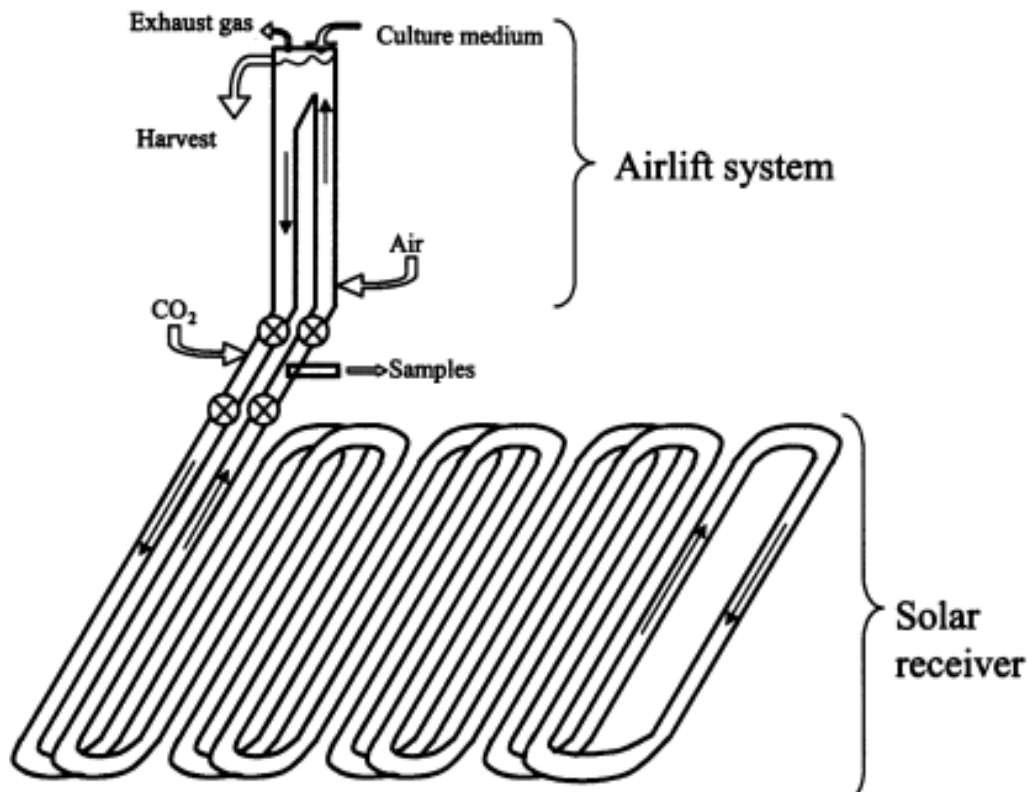
Figure 1.7 - A tubular photobioreactor with parallel run horizontal tubes. Source: Molina *et al.*, (2001)

Fully-closed PBR also provide opportunities for axenic single species cultures that grow in less extreme environments. In addition, there is reduced water loss by evaporation and an overall superior level of control of the long term culture system. However, production is not without its challenges, such as high capital start-up, overall operational costs and maintenance in the form of periodic cleaning due to microbial biofilm formation, whilst still maintaining maximum light exposure (Tercero *et al.*, 2014).

1.3.3.3 Heterotrophic production

Heterotrophic microalgal species utilize organic compounds (e.g., glucose, acetate, glycerol) as both energy and carbon sources in total darkness, but not all algal species are capable of growth using organic substrates as the sole energy source and in the absence of sunlight (Jia *et al.*, 2014). Existing knowledge of fermentation has enabled readily available technology to be adapted for the production of microalgae with complete independence from any environmental conditions and influence and a very high degree of control within the production process (Brennan and Owende, 2010).

Figure 1.8 Modified brewery fermentation towers used for heterotrophic algae biomass production, some of the largest in the world – Kentucky, USA.

Source: http://www.siteselection.com/LifeSciences/2011/sep/nutrition.cfm.

This algal cultivation method is well established and fully accepted as achieving higher volumetric biomass concentrations than photoautotroph models (Liang, 2013). However, there is a need for continuing research and development to overcome the very high cost

hurdle in start-up in order to make heterotrophic culture economically feasible (Huang *et al.*, 2010). At least three other challenges need to be overcome: (1) finding of low or zero value carbon sources to support heterotrophic microalgal growth, (2) design of bioreactors appropriate for industrial scale heterotrophic cultivation, and (3) identification of suitable feedstocks that do not compete with human and animal requirements or other biofuel technologies (Huang *et al.*, 2010).

1.3.3.4 Mixotrophic production

Mixotrophic cultivation of microalgae takes advantage of their ability to utilise organic energy and carbon substrates and perform photosynthesis concurrently (Lee, 2001) and production could assist in the optimisation of medium composition, cultivation conditions and lipid production (Cheirsilp and Torpee, 2012).
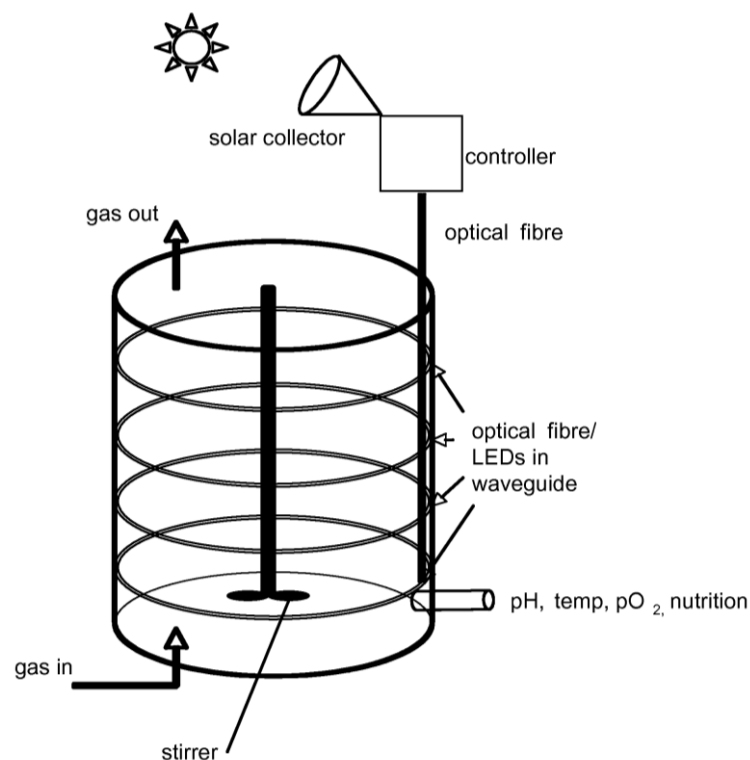
Figure 1.9 Method for cultivating microalgae capable of mixotrophic growth, using photoautotrophs and heterotrophic cultivation controlled nutrient inputs and illumination - Patent application. Source: http://www.google.com/patents/US20120171733.

This combination of technology and advancement in scientific understanding continues to progress and many studies are documented for oleaginous species (Ip *et al.*, 2004; Goksan *et al.*, 2010; Das *et al.*, 2011; Wang *et al.*, 2014) as having increased lipid production. However, a fundamental issue in the optimization of microalgal cultivation systems operated in a mixotrophic regime is the need for a detailed analysis of the effect of the concentration of organic carbon and nitrogen (Pagnanelli *et al.*, 2014).

1.3.4 Harvesting methods

After cultivation, the microalgae biomass has to be separated from its growth medium and recovered with the choice of harvesting techniques dependent upon characteristics of the microalgae, e.g. size, density and the value of the target products (Phukan *et al.*, 2011; Wang *et al.*, 2014). The downstream harvesting and dewatering steps can account for as much as 20 – 30% of the total biomass production costs (Dassey and Theegala, 2013; Chen *et al.*, 2015), mainly due to the dilute nature of microalgae cultures; these factors present a significant challenge that needs to be addressed (Uduman *et al.*, 2010).

Algal recovery requires a two-step process with varying advantages and disadvantages, at differing economic levels. Firstly, bulk harvesting, the primary harvest, involves the concentration of the dilute microalgae suspension into a slurry or paste by use of sedimentation, flocculation, or flotation depending upon the target process objective, followed by a dewatering process known as "thickening", to concentrate the slurry or paste further by use of centrifugation or filtration (Brennan and Owende, 2010). The latter is generally a more energy intensive step than bulk harvesting. The optimal harvesting solution is driven by a variety of factors including algae strain, size, stage of growth, shape, and target product stream (Gerardo *et al.*, 2015).

Significant harvesting costs arise because obtaining the algal biomass from the relatively dilute culture requires processing large volumes of water (Dassey and Theegala, 2013). In many commercial systems, the density of harvested algal cultures generally ranges between 80 and 250 mg of dry weight per litre (Grima *et al.*, 2003). Dassey and Theegala, (2013) stated that the theoretical harvesting costs can be lowered significantly with increasing culture densities, by sacrificing biomass harvesting efficiencies for greater process volumes with lower energy consumptions, commercial scale algal plant can develop a cost effective harvesting strategy. The harvested biomass slurry or paste is perishable and must be processed rapidly after harvest and drying is commonly used to extend the viability depending on the final product required (Grima *et al.*, 2003).

1.3.4.1 Drying, extraction and purification of microalgae for food

After dewatering processes have been carried out, drying is required to drive down the final percentage of moisture to approximately <5% (Show *et al.*, 2015); this converts the algal biomass to a stable storable product that can later be subjected to various treatments to yield high value end products (β-carotene, polysaccharides etc.) (Cuellar-Bermudez *et al.*, 2015). The final stage of lipid extraction by cell disruption to obtain the lipid component for biofuel production can then be carried out (Rios *et al.*, 2013). Methods that have been used include sun drying, low-pressure shelf drying, spray drying, drum drying, fluidised bed drying, and freeze drying (see Table 1.7, overleaf).

Table 1.7 Brief overview of drying methods utilized in biomass dehydration processes.

Drying Method	Brief overview	Reference
Sun drying	Sun drying is one of the oldest methods for food preservation and is still used today especially in developing countries. It is difficult to maintain the quality of the end product suitable for human consumption with traditional open sun drying methods. Besides other factors, the slow drying rate due to low temperature is the main cause of biomass degradation and consequential rise in bacterial count.	(Brennan and Owende, 2010; Guldhe *et al.*, 2014)
Low-pressure shelf drying	This process is cheaper than drum drying and more rapid than sun drying. In this method the cell wall of *Chlorella* and *Scenedesmus* sp. cannot be broken.	(Show *et al.*, 2015)
Spray drying	Spray drying is the preferred method of drying high value micro-algal products. Drying is accomplished within a few seconds without denaturing of protein structure and deemed safe for human consumption. Although it is reported that there is significant deterioration of some algal components, such as the pigments.	(Oliveira *et al.*, 2009; Chen *et al.*, 2015)
Drum drying	Uses a sloped rotating cylinder or drum, to move the material being dried from one end to the other by gravity. Drying the algae on the drum dryer has the dual advantage of sterilizing the samples and breaking the cell wall.	(Saleh *et al.*, 1985; Milledge and Heaven, 2013; Show *et al.*, 2015)
Fluidized bed drying	Fluidized-bed drying is a common method to dry particulate materials, such as grain and fruits, due to its excellent heat and mass transfer efficiencies, short drying time, high quality of products, and short reconstitution time.	(Temple and van Boxtel, 1999; Brennan and Owende, 2010),
Freeze drying	Freeze drying is a gentle process in which all the cell constituents are preserved without rupturing the cell wall. Freeze-drying has been widely used for drying micro algae in research laboratories. Freeze-drying is too expensive for use in large-scale commercial recovery.	(Guldhe *et al.*, 2014)

1.3.4.2 Extraction for biofuels

After drying, cell disruption is a key factor in wet oil extraction to facilitate the recovery of intracellular products from microalgae. Extraction methods can be broadly categorised into two approaches depending on the microalgae cell wall integrity and on the product nature to be obtained (Wang *et al.*, 2015). 1) Mechanical disruption; includes pressing, bead-milling, high pressure homogenisation, ultrasonic-assisted extraction and autoclaving and is generally considered preferable as it avoids chemical contamination and preserves the functionality of the cell contents 2) non-mechanical/ chemical extraction ; such as Soxhlet or hexane solvent methods (Barros *et al.*, 2015). Extraction of lipids from microalgal biomass underwent hundreds of optimization studies. However, a return to the accepted soxhlet method is employed as a routine benchmark method for comparisons (Derakhshan *et al.*, 2015)

The cost of dehydration, when added to the cost of algal growth and harvesting, has formed an economic barrier to the mass production of algal powder produced for low-cost products, such as protein or feed. However, after extraction of oils and high value products a further possible high value, protein rich algal cake (meal) remains. This by-product may be utilized within feeds for aquaculture species as an alternative to marine resources and anti-nutritional factor carrying plant based proteins (Hasan, 2001).

1.4 Tilapia

1.4.1 Overview

The family Cichlidae of the order Perciformes are composed of two major lineages, the haplochromines and the tilapiines (Nagl *et al.*, 2001). Tilapiines are tropical fish endemic to freshwater in Africa, Jordan, and Israel (Zengeya *et al.*, 2015). They are possibly one of the first fish species to be cultured, with illustrations found in tombs depicting ancient Egyptians catching fish from ponds and fish being held in cages waiting to be taken to market, suggesting that tilapia were being farmed in Egypt about 2500 BC (Quansah *et al.*, 2007). The genus *Tilapia* was introduced in 1840 by Andrew Smith, with the term "Tilapia" commonly applied as the generic name to a group of cichlids endemic to Africa, even though they may belong to other genera. In the first major and comprehensive reclassification scheme developed by Trewavas, (1983), the several hundred species of *"Tilapia"* were characterised into three major genera based upon the classical approach, which is based on the use of morphological and behavioural characters: *Oreochromis*, the maternal mouth brooders (31 species); *Sarotherodon*, the bi-parental and paternal mouth brooders (9 species); and *Tilapia*, the substrate spawners (Trewavas, 1983). In addition to these major genera, several other related genera were classified to include *Danakilia, Tristromella* with multiple subgenres (Trewavas, 1983). The distinction of species within these tilapiine genera is notoriously difficult, Nagl (2001) attempted to remedy this situation by molecular phylogenetic analysis of tilapiine fishes by analyses of mitochondrial DNA, confirming findings of Trewavas (1983) in previous studies of the differentiation of separate genera listed above. No study has yet included a fully representative taxon sampling of all previously suggested Tilapia related genera and subgenera, nor is a taxonomically valid

classification integrating morphological and molecular data for this key group available (Dunz and Schliewen, 2013).

Of these genera, *Oreochromis* has emerged as the most prominent for the aquaculture industry in the 21st century (Lazard, 2009), this is owing to vast opportunities for protein security, poverty alleviation and economic development in many developing countries (Tacon *et al.*, 2010). Within this genera the species *Oreochromis niloticus,* Nile tilapia (Linnaeus, 1758), has the potential of becoming the most important aquaculture species in the world due to its performance under typical culture conditions, with a high tolerance to a wide range of environmental parameters and adverse management stress conditions, as well as positive characteristics that include fast growth, short food chain, efficient feed conversion ratios and high resistance to disease (Tsadik and Bart, 2007). Although, these biological characteristics are particularly suited to the farming of fish, over the last twenty years, this species have been introduced to international markets worldwide with very limited amount of genetic improvement (Ponzoni *et al.*, 2011).

In response to the inadequate supply of tilapia seed and the deteriorating performance of the fish in many aquaculture systems in Asia, scientists from the International Centre for Living Aquatic Resources Management (ICLARM), now known as the World Fish Centre, started the development of Genetically Improved Farmed Tilapia (GIFT).

The objectives of the project may be summarised as follows.

1) To maintain and continuously improve the GIFT strain of Nile tilapia and to distribute it to partner countries that are likely to benefit from its use.

2) To conduct research that may enhance the effectiveness of the genetic improvement program (e.g. refining the methodology with respect to management of inbreeding and

effective population size, introducing new traits such as fillet yield, flesh quality and response to thermal treatment to the breeding objective)(Ponzoni *et al.*, 2011). With one main aim of dramatically increasing aquaculture yields via a programme of selective breeding of several strains of Nile tilapia, without the need for any application of controversial biotechnology or genetic modification (WorldFish, 2004).

1.4.2 Biology & Diet of Nile tilapia

1.4.2.1 Biology & Diet

Generally, tilapia are herbivorous, able to obtain substantial nutritional benefit from plant matter and/or detritus of plant origin (Njiru *et al.*, 2004). Processing filamentous and planktonic algae via structural adaptions such as nutrient absorption along the length of a long, coiled intestine (usually at least six times the total length of the fish), sharp pharyngeal teeth for physical grinding of plant tissue, otherwise, an increased number of long, thin, closely spaced gill rakers (for filter feeding) and a stomach pH below 2 (ruptures the cell walls of blue-green algae and bacteria) (Popma and Masser, 1999).

This means that tilapia cannot consume large amounts of food in a short time period, as carnivorous species do (Beveridge and Baird, 2000). Adult tilapia are predominantly herbivorous, known to feed on phytoplankton, aquatic plants, benthic fauna, detritus and graze on submerged bacterial films (periphyton)(Rondel *et al.*, 2008). They are also opportunistic omnivores consuming insects and small invertebrates such as crustacea, with several researchers reporting cannibalism among cultured tilapia (Borges *et al.*, 2005; Fessehaye *et al.*, 2006). Juveniles consume a more omnivorous diet consisting primarily of crustacea and phytoplankton (Jauncey, 1998b), and all life stages exhibit diurnal feeding patterns.

1.4.3 Global supply and trade

Nile tilapia, *Oreochromis niloticus* grow to a maximum length of 60 - 70 cm, weighing approximately 3 – 5 kg (FAO, 2016). The average global market size of whole *O. niloticus* is an approximate weight of 600g (Thodesen and Ponzoni, 2004).

In terms of production volume, Nile tilapia are currently the second most abundantly farmed fish worldwide, representing 6.3% of overall global aquaculture production (FAO, 2011) with global production volumes increasing from just over 100,000 mt in 1980 to approximately 4 million mt in 2013 , and the industry has an estimated total value of US $ 6.7 billion (Lian Heinhuis, 2015).
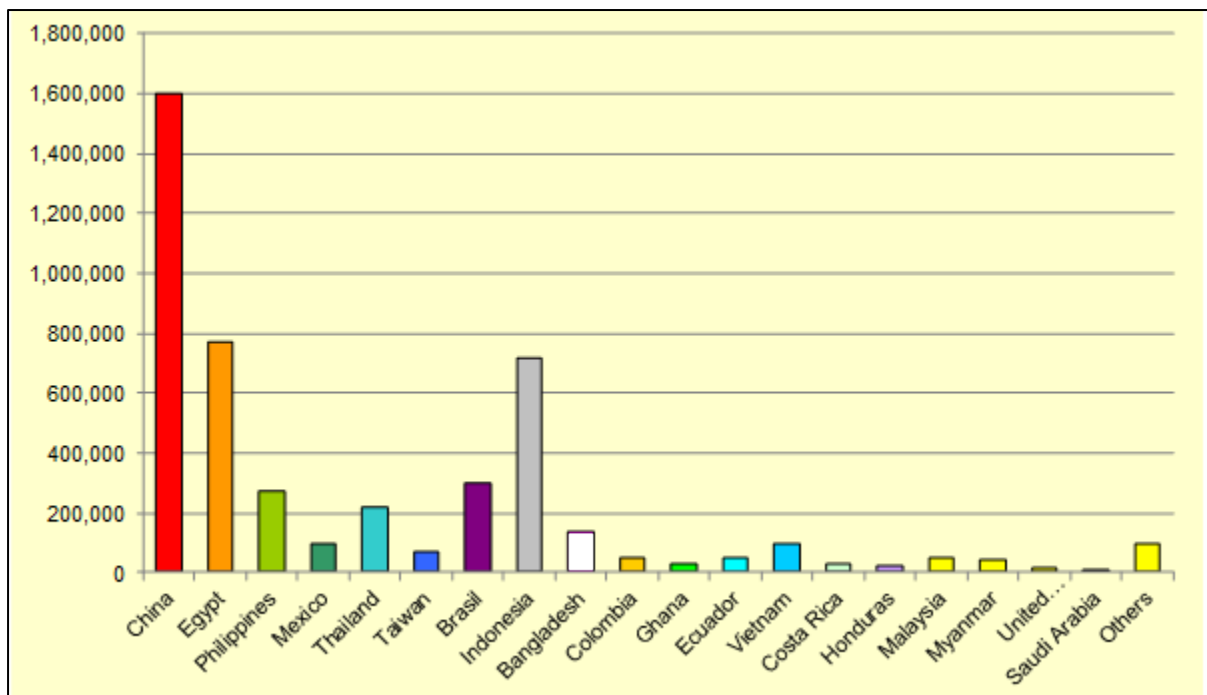
Figure 1.10 Global aquaculture production by country of *O. niloticus* in 2012.

Source: (FAO, 2012) .

Global production of farmed tilapia is wide spread with production statistics for 135 countries and territories on all continents recorded by the FAO (2014), the main

contributing aquaculture production countries of *O. niloticus* in 2012 were China, Egypt, Indonesia and Brazil (see Figure 1.11 above). Professor Kevin Fitzsimmons at the INFOFISH Tilapia conference 2015 estimated that global tilapia production exceeded 4.85 million tonnes in 2014 with a forecasted growth of 6% in 2015 to 5 million tonnes (FAO/GLOBEFISH, 2015). The export market is currently dominated by China that maintained its position as the single largest producer, with approximately 1.6 million tonnes of production and 403 thousand tonnes of export in 2013 (FAO/GLOBEFISH, 2015), while the United States is the biggest importer of Chinese tilapia, totalling 168 thousand tonnes imported in 2012(FAO, 2012). This included 144 thousand tonnes of frozen fillets worth $644 million and 24 thousand tonnes of whole frozen tilapia worth $47 million (FAO, 2012). With China supplying approximately 74 percent of all tilapia (fresh and frozen) imported into the United States (FAO, 2012). Additional expansion also continued from smaller countries with more farms and more productivity in Egypt, Indonesia and Brazil. Tilapias are grown utilising various techniques/ approaches and through differing production strategies, ranging from rural subsistence (low input practices, for non-commercial and household consumption) to a largescale (capital intensive, commercial purposes) level, depending on the intensity of management and inputs employed (Rafael, 2008).

1.5 Research aims and objectives

1.5.1 Aims

The broader aims of this research program were to:

- Test the hypothesis that a significant level of a plant protein concentrate, (i.e. soya bean meal) can be replaced with a commercial algae source whilst maintaining the nutritional requirements of tilapia.

- Determine whether specific algal biomass can adequately support growth and feed utilization to the same degree as a typical reference diet for this species; also to confirm algae inclusion thresholds.

- Test the hypothesis that algae inclusion can impact functionality thereby promoting enhanced gut integrity and improve liver morphology compared to control diets without algae.

- Compare whether tilapia fed algae enriched diets can be effectively pigmented with respect to integument colouration due to the presence of carotenoids in algae.

1.5.2 Research objectives

The principle objectives within each experimental feeding trial conducted on tilapia were:

1. To identify and source bespoke strains of micro-algal bio-mass (with extracted oil) for inclusion in scientifically formulated diets for juvenile tilapia.

2. Provide a comprehensive nutritional profile for each algal biomass sample and compare with standard raw materials and ingredients suited for tilapia diets.

3. Design and implement successive feeding trials over a defined (8 week) time course to compare two types of algae products at incremental inclusion levels to determine maximum limits for their inclusion.

4. Obtain growth performance and feed utilization data such as SGR &FCR, body composition profile for comparison with the scientific literature.

5. The objective of developing a reliable protocol to measure the degree of integument pigmentation due to algal enrichment using image analysis techniques.

To provide supporting evidence based on histological methods of the influence of algal inclusion on specific gut integrity parameters (mucosal fold dimensions and assortative area) and also hepatic health based on liver morphology and appearance.

Chapter 2

Assessment of commercial proprietary
Nannochloropsis sp. in diets for
Oreochromis niloticus

2.1 Introduction

Nannochloropsis belongs to the class of Eustigmatophytes (Hibberd and Leedale, 1970), which are predominantly marine unicellular coccoid photosynthetic organisms. Eustigmatophytes are comparable to other economically and ecologically important classes like diatoms and brown algae classified in the phylum heterokontophyta (Hibberd and Leedale, 1970). Hibberd (1981), described six known species within the genus *Nannochloropsis* (*N. gaditana, N. granulate, N. limnetica, N. oceanic, N. oculata* and *N. salina)* and reported that these cells are typically small in size (2-4 µm in diameter), spherical to slightly ovoid, non-flagellate and unicellular with the endoplasmic reticulum continuous within the nuclear envelope of the chloroplast. *Nannochloropsis* spp*.* have one single chloroplast containing several bands of photosynthetic lamellae contained within a polysaccharide cell wall of cellulose (Carpinelli *et al.*, 2014), an organelle which is identical to all other photosynthetic chloroplast membrane-bound plastids, in that each cell has the capacity to accumulate a range of valuable pigments, such as chlorophyll and various carotenoids (that are insoluble in water). *Nannochloropsis* spp are also reported to build up high concentrations of a range of pigments such astaxanthin, zeaxanthin and canthaxanthin (Lubian *et al.*, 2000), with only chlorophyll *a* found within *Nannochloropsis* cells, completely lacking chlorophylls *b* and *c*, within the chloroplast. This chlorophyll *a* is the primary pigment with which algae and higher plants capture the energy of the sun through the absorption of certain wavelengths of light for the process of photosynthesis and lipid production (Brown, 1987).

The genus *Nannochloropsis* has come into focus as a potential feedstock for biofuel production; it consists predominantly of marine microalga and species within the genus are

often rich in TAGs (Hu *et al.*, 2008), which function as the main energy storage compound in cells (Sukenik and Carmeli, 1990).

TAGs accumulate during periods of illumination, with cellular content further increased during stress conditions, such as excessive light regimes or salinity changes. Nitrogen deprivation is reported to increase the lipid content to 50% of the dry weight of the cells, along with high productivity (Rodolfi *et al.*, 2009; Kilian *et al.*, 2011; Pal *et al.*, 2011). Furthermore, whilst krill and GM crops are excellent sources of PUFAs, *Nannochloropsis* might be the most promising alternative to these sources, as one of the primary producers of EPA (Boussiba *et al.*, 1987; Babuskin *et al.*, 2014; Ryckebosch *et al.*, 2014).

The use of *Nannochloropsis* in aquaculture has been widely described (Faulk and Holt, 2005; Rocha *et al.*, 2008; Ferreira *et al.*, 2009; Zaki and Saad, 2010; Chen *et al.*, 2012), as it contains all the substances of biological value required by aquaculture species (e.g. lipids, PUFAs, amino acids, antioxidants and pigments (carotenoids), vitamins and minerals). *Nannochloropsis* has primarily been used as an energy-rich food source for fish larvae and rotifer nutrition in "green water techniques" and has also been reported to stabilize water quality and provide shielding for light-sensitive fish larvae (Rocha *et al.*, 2008) as well as having possible antibacterial activity (Kokou *et al.*, 2012).

The present study involved low levels of inclusion of a commercial Nannochloropsis sp. meal within feeds for *O. niloticus* by substitution of soyabean meal. Corn starch and corn oil levels varied to maintain feeds that were approximately isonitrogenous and isoenergetic.

2.2 Materials & Methods

2.2.1 Microalgae production and characterisation

A 2275 g sample of *Nannochloropsis* sp. (Heliae® Lot Number: 0512A 002, Growth Phase, Product ID: HNCW0GAFD00) was acquired from Heliae®, Arizona, USA. This strain is cultured by the supplier in indoor proprietary enclosed photo-bioreactors (PBR) until sufficient mass is accumulated to culture in an outdoor PBR. Both the indoor and outdoor culturing processes are sustainable through using recirculating media, requiring only the addition of salt, inorganic nutrients and carbon dioxide. The biomass was harvested, free of chemicals or flocculants, and dewatered via a patented centrifuge technology until the biomass had most of the free water removed. The biomass then had selective components extracted (i.e. lipids), before being freeze dried, packaged and sent for analysis to the Food and Nutrition Analysis Unit, Plymouth University, Devon, U.K.

Scanning electron microscopy observations were carried out to explore the ultrastructure of the microalgae supplied (Figure 2.1, below). Preparation was carried out as described in Section 2.2.6.2.2.
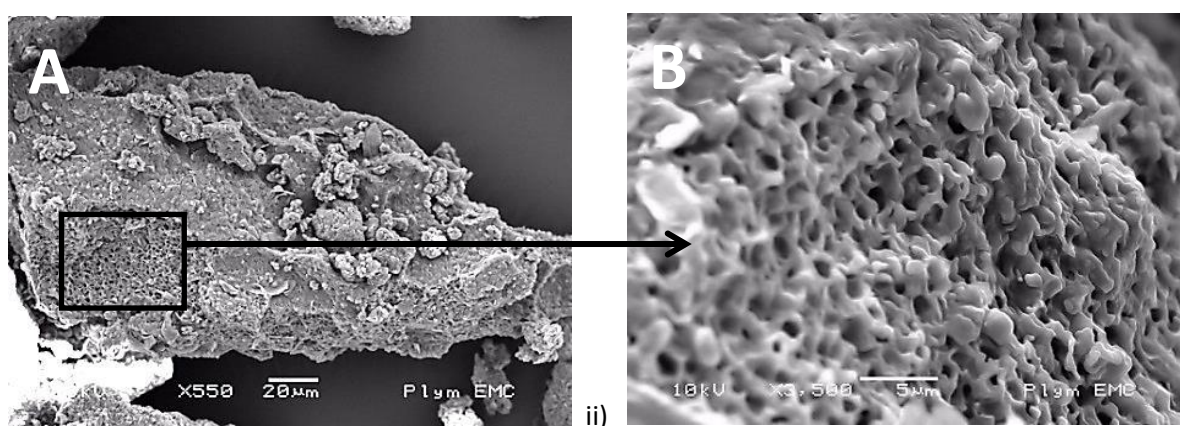
Figure 2.1 Scanning electron micrographs of Heliae™ 0512A 001 *Nannochloropsis* sp. showing fragmented structure with honeycomb ultrastructure. A) Scale bar = 20 μm at and B) Scale bar = 5 μm.

Proximate compositional analysis of the product was also conducted (Table 2.1) using wet chemistry methods as described in section 2.2.5 using standard protocols (AOAC, 2012).

Table 2.1 Proximate composition of *Nannochloropsis* sp. (dry matter basis).

Composition	%
Protein	26.77
Ash	8.02
Lipid	5.82
NFE + fibre	59.39
Gross energy	19.34 (MJ kg^{-1})

NFE = (Nitrogen-free extracts)

2.2.2 Feed formulation and diet preparation

Experimental diets were prepared in 5 kg batches. Four iso-nitrogenous and iso-lipidic diets were formulated (Table 2.2) according to guidelines and specifications for tilapia (Jauncey, 1998a) using Feedsoft Professional® 3.0, Feedsoft™, UK.

Table 2.2 Dietary formulation & proximate composition of experimental diets.

Ingredients g kg^{-1}	Control	5%	10%	15%
Herring meal [a] (LT94)	200	200	200	200
Corn Oil [b]	47.41	45.09	42.77	40.44
Maize (corn) gluten [c]	50	50	50	50
Soya Bean meal [d] (hamlet HP100)	321.18	297.69	274.21	250.73
Corn Starch [e]	371.41	347.22	323.02	298.83
CMC – Binder [f]	5	5	5	5
Vitamin-mineral premix [g]	5	5	5	5
Nannochloropsis [h]	-	50	100	150
Total (g)	1000	1000	1000	1000
Proximate Analysis (% dry matter basis)				
Crude protein (N x6.25) [i]	36.87 ± 0.34	35.95 ± 0.40	36.85 ± 0.66	36.68 ± 0.58
Lipid [i]	7.24 ± 0.07	8.41 ± 0.06	9.36 ± 0.02	9.97 ± 0.06
Ash [i]	5.20 ± 0.04	5.30 ± 0.14	5.41 ± 0.02	5.53 ± 0.08
NFE [j]	43.93	43.02	41.72	41.54
Gross energy (MJ kg^{-1})	19.25 ± 0.03	19.54 ± 0.01	19.64 ± 0.06	20.03 ± 0.02

1
2 [a] CC Moore, Dorset, UK
3 [b] Corn Oil, Sainsbury, UK
4 [c] Roquette Frères, France
5 [d] Hamlet Protein, Denmark
6 [e] Sigma-Aldrich Ltd, UK
7 [f] CMC (Carboxymethyl cellulose), Sigma-
8 Aldrich Ltd, UK
9

10
11 [g] Premier Nutrition - vitamin/mineral
12 premix, UK
13 [h] Heliae®, Arizona, USA
14 [i] Values are given based on a dry matter
15 basis
16 [j] NFE (Nitrogen-free extracts) = dry
17 matter – crude protein + crude lipid + ash

Dry milled ingredients were weighed into a plastic container and mixed uniformly to ensure homogenous distribution of the dietary components. The dry blended ingredients were then mixed for approximately 30 minutes in a Hobart food mixer (Hobart Food Equipment, Australia). Corn oil was then added gradually in a continuous flow, after further mixing, warm water was added to the mixture to form a consistency suitable for cold press extrusion (La Monferrina P6, La Monferrina, Asti, Italy) to produce 2 mm strands. The resulting strands (see Figure 2.2) were carefully laid out into cleaned polypropylene trays lined with tin foil. These trays were subsequently transferred to a warm air oven (Genlab, MINO/200/SS/F, Cheshire, UK) and were left to dry at 40 °C for 48 hours, turning approximately every 12 hours. Diets were cooled and crushed to a suitable size for juvenile tilapia (Figure 2.3), placed into plastic vessels that had been previously sterilized with 70% Industrial Methylated Spirits (IMS, v/v in water), labelled and kept in a dark, refrigerated environment (5 °C) until required.

Figure 2.2 Extrusion of 2mm strands of formulated diet though brass die prior to drying.
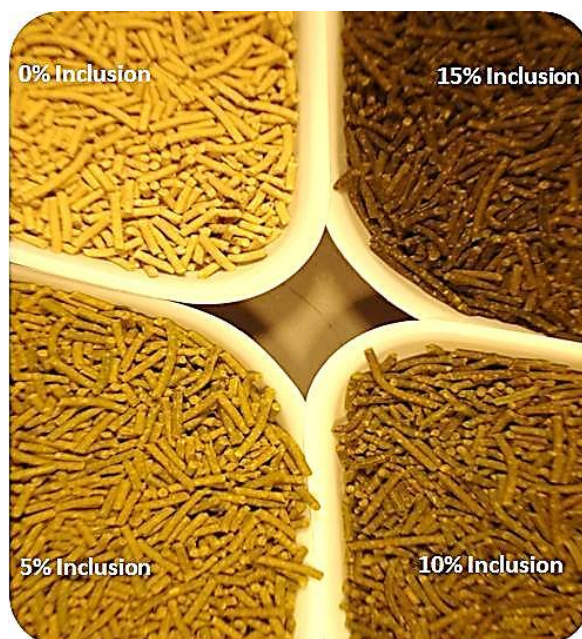
Figure 2.3 Pelleted feed produced in the Feed Production Centre, Aquaculture Health and Nutrition Unit, Plymouth University.

2.2.3 System set up and experimental design

A group of 500 monosex male (XY genotype) Nile tilapia (*Oreochromis niloticus*) were purchased from Stirling University, UK into the Aquaculture, Health and Nutrition Research Facility at Plymouth University, U.K. An acclimation period of 8 weeks followed to take the individual weight of the newly arrived fish from 0.02 g to 15 g approx. start weight. During this period, feeding was carried out 8 - 10 times daily to approximately 30 % of the total biomass, according to recommended feeding tables (Jauncey, 1998a), using a fine grade commercial pellet (EWOS Micro 015P – 5 P). Fish were carefully selected so as to achieve an overall distribution of 35 fish per tank with unit weights within 3% for each of the twelve fibreglass tanks. The 80 litre experimental fibreglass tanks form part of a closed recirculation system (see Figure 2.4), which was aerated and kept at a controlled temperature of 27°C ± 0.5 °C provided by a 3KW inline heater (Electro Titanium Aquatic heater). A control group

and three experimental diet groups of 5%, 10% and 15% algal inclusion therefore provided four treatment groups of fish, with each treatment group triplicated for the purposes of statistical analysis (three tanks per treatment). Prior to starting the trial, each unit (tank) of fish was accurately bulk weighed using RADWAG 15 kg waterproof single load cell platform scales (RADWAG Wagi Elektroniczne, Poland). Each tank of fish was fed a 3% ration, three times daily at the same time each day, with half ration on Sunday prior to biweekly weighing. A photoperiod of 12 h light /12 h dark was provided throughout the trial by an automated fluorescent lamp system. Water parameters were monitored using a Hach Lange (DR2800) Laboratory Spectrophotometer throughout the trial period. Ammonium (0.03 – 0.09 mg L^{-1}), nitrite (0.02 – 0.1 mg L^{-1}) & nitrate (< 30 mg L^{-1}) levels were monitored weekly and dissolved oxygen (6 - 7 mg L^{-1}) and pH (6 - 7) levels monitored daily throughout the trial period, with all falling within the accepted range for this species (Popma and Masse, 1999). The trial was concluded at day 68 of feeding, with sampling conducted on this final day.

Figure 2.4 Plymouth University Closed Recirculation System - utilized during trial period.

2.2.4 Growth and feed utilization calculations

Growth performance and feed utilization parameters were determined using the following equations:

Parameter	Equation
Weight Gain (WG g fish^{-1})	FbW - IbW
Specific Growth Rate (SGR, % d^{-1})	$\dfrac{(Ln)\ FbW - (Ln)\ IbW}{t}$ x 100
Feed Conversion Ratio (FCR, g)	TfI / (Wf - Wo)
Hepatosomatic Index (HSI)	(LW / FbW) x 100
Condition Factor (K %)	(FbW x 100) / FL^3
Survival (%)	100 – Mortality (%) = (final Nb / initial Nb) × 100

Where:

(Ln) FbW = Natural logarithm of the final body weight

(Ln) IbW = Natural logarithm of the initial body weight

FbW = Final body Weight (g)

IbW = Initial body Weight (g)

TfI= Total feed Intake (g), WG = Weight gain (g)

FL = Final Length (cm), T=Time (day)

PI= Protein intake (g)

LW= Liver Weight

Initial Nb = initial number of fish

Final Nb = final number of fish

2.2.5 Chemical and Proximate analysis

Dried samples of raw ingredients, experimental diets and fish tissue were all subjected to analysis for the determination of moisture (water content), ash, crude proteins, crude lipids and gross energy content. Fish were sampled at the beginning and at the end of the trial to determine carcass composition. Samples were all analysed in triplicate (unless otherwise stated) in accordance with AOAC (2012) guidelines.

2.2.5.1 Protein

Determination of crude protein (CP) of feed ingredients, diets and whole fish carcass was performed by the Kjeldahl method (AOAC, 2012), to determine the total nitrogen (N) content. This value was then multiplied by a conversion factor of 6.25 to calculate the CP content.

$$\% \text{ Nitrogen} = \frac{(\text{ml sample titrant} - \text{ml blank titrant}) \text{ x Acid normality x MW Nitrogen}}{\text{Sample Weight (g)}}$$

Where: Acid normality (N) = 0.2, MW of Nitrogen = 14.01 g

$$\% \text{ Protein} = \% \text{ Nitrogen x Conversion Factor}$$

$$\text{Conversion Factor} = 6.25$$

2.2.5.2 Ash

Ash (total mineral or inorganic) content was determined by adding a known weight of sample (500 mg ± 100 mg) to a pre-weighed crucible. The crucibles were then incinerated in

a muffle furnace (Carbolite, Sheffield, England) at 550 °C for 8 hours, until light grey ash results or to constant weight. Samples were cooled in a desiccator and weighed soon after reaching room temperature. Percentage ash was determined from the sample residue by the following calculation:

$$\% \text{ Ash} = \frac{(\text{Weight of crucible (g)}+ \text{Residue(g)}) - \text{weight of crucible (g)}}{\text{Sample weight (g)}} \text{ x } 100$$

2.2.5.3 Lipid

Lipid content was determined by using Rapid Soxhlet extraction method. For accurate analysis, bare hands were avoided when handling glassware at all times (either tongs or protective gloves were used as appropriate). A known weight of sample (approx. 3 g weighed to 0.1mg) was added to a cellulose thimble and plugged loosely with a cotton wool bung. Thimbles in thimble holders were then inserted into pre-weighed extraction beakers containing anti-bumping granules then flooded with 140ml petroleum ether 40–60°C. The extraction beakers were inserted into the Soxtec™ extraction system (Tecator Systems, Högnäs, Sweden; Model Soxtec 1043, Service unit 1046; see Figure 2.6) and the correct program was selected. At the end of the program the beakers were removed from the unit and returned to the rack in sequence and then moved to a fume cupboard until all traces of solvent had evaporated. Beakers were re-weighed on same three decimal place balance and lipid content was determined as:

$$\% \text{ Lipid} = \frac{\text{Final weight of beaker (g)} - \text{Initial weight of beaker (g)}}{\text{Initial weight of sample (g)}} \text{ x } 100$$

2.2.5.4 Moisture

All samples were weighed and then dried at 105 °C in a fan assisted oven (Gallenkamp Oven BS, Model – OV-160, U.K.) until a constant weight was achieved.

Moisture content was calculated as:

$$\% \text{ Moisture} = \frac{W2 - W3}{W2 - W1} \times 100$$

% Dry Weight = wet weight x (100/ 100 - % Moisture)

W1: Initial weight of empty dish/crucible (g)

W2: Weight of crucible+ food before drying (g)

W3: Final weight of crucible + food after drying (g)

2.2.5.5 Gross energy

After calibration of the device using thermochemical standardised Benzoic acid pellets, gross energy was determined on duplicate samples by means of Bomb-Calorimetry (Adiabatic Bomb-Calorimeter, Model 1356, Parr Instrument Company, IL, USA). Whereby heat created by a sample, burned under an oxygen atmosphere in a closed submerged vessel, is measured. The ground and dried sample was first compressed into a 1 g (+/- 0.01g) pellet and weighed on a four decimal balance. This was then placed inside a stainless steel container (the 'Bomb' or 'decomposition vessel') in a crucible of known weight along with 1 ml of distilled water into the bomb cylinder. A 10cm length of fuse wire was clipped to the electrodes and almost touching the surface of the pellet. The bomb head was then placed carefully into the bomb cylinder that contained 2000 g (± 0.5g) of distilled water. The lid was placed on and hand-tightened. The chamber was then filled with oxygen (to 30 bar or 435

PSI). The sample was then electrically ignited through the wire thread inside. During the combustion the core temperature in the crucible is approximately 1000 °C. All organic matter is oxidized under these conditions and the heat transfer to the surrounding water jacket is detected. The internal microprocessor of the bomb calorimeter converts this information into a specific energy value of megajoules per kilogram (MJ kg^{-1}).

2.2.6 Sampling

Prior to commencement of the nutritional trial, fish, diets and ingredients were collected for chemical analysis. Twelve fish were randomly sampled for carcass composition analysis, prior to distribution as described in section 2.3.3. At the end of the experiment, 3 fish per tank (*n=3* per treatment) were randomly removed from each tank, euthanized with buffered MS222 (tricaine methanesulphonate, Pharmaq) at 200 mg L^{-1} (with sodium bicarbonate at 400 mg L^{-1} for pH buffering) and individually weighed per tank for carcass composition. Fish sampled for haematology were terminally anaesthetised and decerebrated in compliance with the Home Office Animal Scientific Procedures act (1986).

2.2.6.1 Haematology

At the end of the trial, three fish per tank from each of the treatment groups (*n=9*) were anaesthetised (Refer to section above). Blood was sampled from the caudal vein using 25 gauge needles and a 1 ml syringe and the sample placed into 2 ml micro centrifuge tubes.

2.2.6.1.1 Haematocrit

Haematocrit (Hct) values were determined immediately after blood collection. Hct values were calculated by drawing fresh blood into heparinised haematocrit tubes which were then sealed with plasticine and centrifuged at 3600 x g for five mins in a micro-haematocrit centrifuge (1-14 Microfuge, Sigma) as described by Hrubec *et al.* (2000). Hct values were measured using a using a Hawksley tube reader and expressed as percentage packed cell volume (% PCV).

2.2.6.1.2 Haemoglobin (Hb)

Haemoglobin was measured using the cyanmethemoglobin method described by Silverton, (1976). Haemoglobin was determined based on Drabkin's cyanide-ferricyanide solution (Sigma Aldrich Ltd). Five µl of blood was added to 1 ml of Drabkin's solution, vortex mixed immediately and measured using a spectrophotometer at 540 nm (Thermo Fisher Scientific, Spectronic™ 200, USA). Haemoglobin absorbance was measured from a curve prepared from reference standards (cyanmethaemoglobin; Sigma diagnostic kit № 525 A). The values obtained were expressed in g dL^{-1}.

2.2.6.1.3 Blood cell counts

Visual counting of blood cells was as described by Handy and Depledge, (1999). Dacie's solution was prepared using 10 ml of 40 % formaldehyde, 31.1g of trisodium citrate, 1g of crystalline blue (stain), dissolved together in a total volume of one litre of distilled water, which was then passed through a 0.45 µm nylon filter membrane (Sigma-Aldrich, UK). Twenty µl of blood was pipetted into a 2 ml micro centrifuge tube containing 980 µl of Dacie's solution (this provides a 1:50 dilution of blood sample), mixed with a vortex mixer for ~ 60 seconds, and then refrigerated at 4 °C until required.

Ten µl of the blood/ Dacie's solution mixture was then pipetted into a Neubauer counting chamber and allowed to fill the chamber for approximately one minute, as described by Handy and Depledge (1999). The erythrocyte (red blood cell, RBC) and total leukocyte (white blood cell, WBC) counts were determined optically using an x 40 objective, with the following calculation applied to determine the number of cells per defined volume:

$$\text{Total RBC count} = \frac{\text{Average cell count}}{\text{Area (mm3)}} \times 50 \text{ (dilution factor)}$$

To convert to standard units divide by $1 \times 10^6 (\times 10^6 \text{mm}^3)$

$$\text{Total WBC counts} = \frac{\text{Average cell count}}{\text{area(mm3)}} \times 50 \text{ (dilution factor)}$$

To convert to standard units divide by $1 \times 10^3 (\times 10^3 \text{mm}^3)$

2.2.6.1.4 Blood differential cell counts

In order to quantify the circulatory levels of lymphocytes, granulocytes, thrombocytes and monocytes, 5 µl of whole blood was smeared onto microscope slides. Slides were air-dried, fixed in 95% methanol and stained with Wright-Giemsa solution (BDH, Laboratory supplies Poole, UK) before mounting- using Dibutyl-phthalate Xylene (DPX) under a cover slip.

Lymphocytes, granulocytes, thrombocytes and monocytes were identified and counted following the descriptions of Claver and Quaglia (2009). Two hundred cells per sample were counted from three fish per tank (*n = 9* per treatment) and the values were expressed as a percentage of the total leukocyte count.

2.2.6.1.5 Serum lysozyme activity

Blood was left to clot for a period of 12 h (at 4 °C) and then centrifuged at 3600 x g for 5 min to recover the serum, which was then removed and stored at -20 °C until analysis of lysozyme activity as described by Ellis (1990). (See Appendix 1 - Determination of lysozyme activity using a turbidimetric assay). Samples were thawed at room temperature during preparation before measurement.

A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance at 0.001 min^{-1}.

2.2.6.2 Histology (light microscopy, scanning electron microscopy)

Three fish were selected per tank from four treatment groups ($n= 9$ per treatment), and removed for terminal anaesthesia using MS 222 at 200 mg L^{-1} (with sodium bicarbonate at 400 mg L^{-1} for pH buffer) for 5 mins in conjunction with decerebration of brain (in accordance with Home Office – Schedule 1 regulations). All visceral samples were removed simultaneously (see Figure 2.5) and whole weight recorded. The liver was removed and weighed separately for hepatosomatic index (HSI) calculations (see section 2.2.4). In preparation for histological appraisal of mid-intestine and liver, samples were carefully dissected from the removed viscera. All the tissue samples were fixed in 10% formalin (v/v in water).
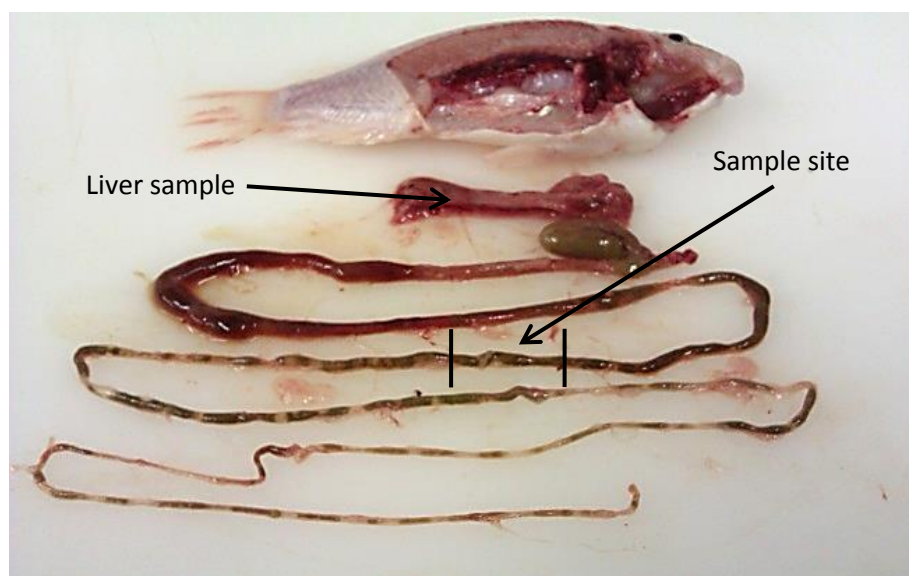
Figure 2.5 Visceral organs removed to establish mid-intestine sample site and liver separation.

2.2.6.2.1 Light Microscopy

After fixing in 10% formalin, samples were dehydrated in graded ethanol concentrations (70%, 80%, 95% & 100%, v/v in water) before equilibration in xylene using a Leica TP1020 automatic tissue processor. Three separate mid-intestinal and liver samples per tank (*n=9* per treatment) were then embedded into paraffin wax blocks for sectioning. From each block, multiple sets of transverse sections (5 μm thick) were prepared using a RM2235 microtome, mounted on slides using albumin and stained with haematoxylin and eosin (H&E) stain using the Leica Autostainer XL, with cover slips fixed in place using DPX. Liver images were analysed for density of hepatocytes of a stained area, expressed as arbitrary units (AU). Images of mid-intestine and liver samples were taken with an Olympus e-620 digital camera mounted on a Vanox AHBT Olympus research microscope.

Slide-mounted mid-intestinal samples were assessed as described by Dimitroglou *et al.,*(2009) for mucosal fold length (FL) and perimeter ratio (PR). Liver blocks were subjected to staining with H & E stain and mounted liver samples were assessed for hepatocyte nuclei density. In addition, abnormal necrosis of cells and hepatocyte integrity were qualitatively assessed. Image J© (Java™) Platform analysis software was used to measure a variety of parameters for each of the stained sections, in which 10 mucosal folds were measured for length from a standardised measurement and the PR determined by measurement of internal perimeter (IP) of the intestinal lumen and the external perimeter (EP) of the intestine (see calculation below).

$$\text{Perimeter Ratio (AU)} = IP/EP.$$

Liver nuclei were counted in four consistently placed 100 μm squares per sample images. By stacking three images, 12 fields of view at x 100 magnification were studied (*n=9* per treatment) as described by Figueiredo-Fernandes *et al.,*(2007).

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2.2.6.2.2 Scanning Electron Microscopy

High magnification SEM micrographs were analysed in order to measure the microvilli density (arbitrary units) as described by Dimitroglou *et al.*, (2009). Three fish per tank (n=9 for each treatment) of 0.5 cm mid-intestinal tubular sections were cut in half. One half was washed for 30 seconds to remove epithelial mucus by holding it in a solution of 1% S-carboxymethyl-l-cysteine (Sigma), which was being constantly agitated on a magnetic stirring plate, before being placed in glass vials with card I.D. labels and fixed in 2.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.2). The glutaraldehyde was removed from the samples 24 hrs later and replaced with an equal volume of 0.1M sodium cacodylate buffer, pH 7.2. Samples were then dehydrated in graded ethanol solutions of 25%, 50%, 75%, 90% and 100% (v/v in water) for 10 mins in each solution.

Mid-intestinal samples and dried algae samples were critical point dried with liquid CO_2 (Emitech K850) as the transition fluid. The dried micro algae sample and all mid-intestinal samples were mounted on stubs using adhesive carbon dots and/or conductive adhesive liquid silver paint, followed by gold sputter-coating using a compact rotary-pumped sputter coater (K550 Emitech). SEM images were obtained using a JSM 6610 LV cryo-electron microscope (JEOL, Japan) at 10 - 15 kV accelerating voltage. Images (magnification ×20,000) were analysed in order to measure the density of the microvilli (MD). In order to calculate the density all images were analysed using Image J (Version 1.36) by inputting and stacking images and implementing four squares of 1 μm with microvilli heads being counted per area (Figure 2.6, overleaf).
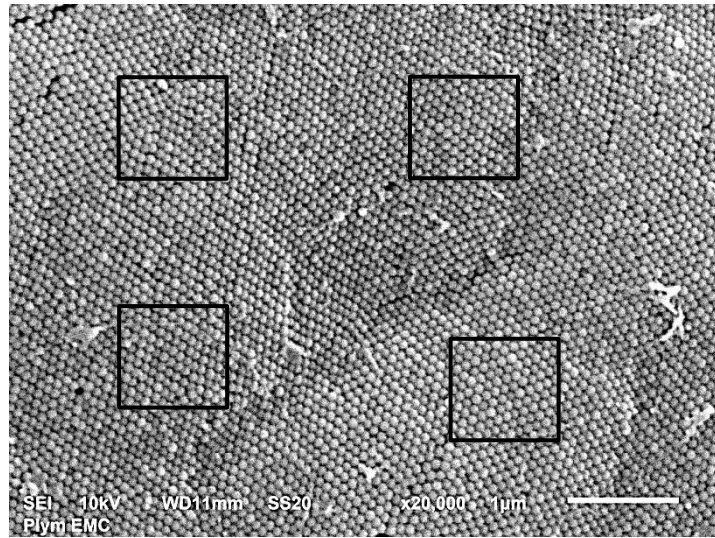
Figure 2.6 Scanning electron micrograph of mid-intestine microvilli sample, with four superimposed 1 μm squares for microvilli head counts using Image J software.

2.2.6.3 Photographic assessment of skin colouration

2.2.6.3.1 Background

The objective of this photographic assessment was to measure the variability of skin colouration in red Nile tilapia as a result of microalgae dietary inclusion. One way to achieve this is to measure surface reflectance using spectrophotometry, which provides precise information on the intensity distribution of wavelengths reflected (Wallat *et al.*, 2005). However, this method can introduce many variables and photographic appraisal can be more rigorous and reliable method of evaluation if the correct measures are implemented as described by Stevens *et al.* (2007).

2.2.6.3.2 Image capture

The image capture protocol was based on a recent study by Stevens *et al.* (2007), which highlighted the challenges associated with using digital photography to study animal colouration.

Two fish were collected from each tank (*n=6* per treatment) and euthanized with buffered 400 mg L^{-1} MS222 (10 minutes) in conjunction with decerebration, prior to image capture. Fish were both individually and collectively photographed by being placed on their left side on a new white chopping board and gently dried with a J-cloth to remove excess water that could affect the RAW image.

A Panasonic Lumix DMC-FZ200 (Leica DC Lens / 25-600 mm) bridge camera was used to ensure that no 'automatic' adjustments of the image were made which might have biased the results (Stevens *et al.*, 2007). The parameters used in image capture are presented in Table 2.3 (overleaf).

Table 2.3 Camera settings used during image capture.

The following camera settings were all specifically adjusted to allow standardisation.

Parameter	Details
Aperture	An aperture value of F8 was chosen to allow as much light as possible to reach the image sensor.
International Standards Organization (ISO) settings	An ISO value of 200 was used. This setting ensured the image sensor of the camera was less sensitive to light providing a much sharper and detailed image. The reduction in image sensor sensitivity was counteracted by the subject being adequately lit as described below.
White balance	A white balance specific to the incandescent bulbs used to light the fish was selected.
Focus	All images were focused manually by the operator and set on C1 – Custom Settings
File type	All images were saved as RAW (unprocessed) files as this file type contains all the original image information, unlike JPEGs which compress images, losing colour and skewing the image data.

In order to ensure that standardised light conditions were used throughout the experimental period, digital photographs were taken inside a room with no natural light. This ensured that both the quantity and type of light could be fully controlled. A lighting rig with two 60 W incandescent bulbs (Canon™) fixed in position gave a constant level of lighting eliminating the need for a flash. Camera position in relation to the fish was also standardised using an attachment to the lighting rig which ensured the camera was 60 cm away from each fish (Figure 2.7, overleaf).

All image capture was carried out on the same day and the process of calibration was conducted at the start of the sample session to ensure that variations in light or camera bias would not affect comparisons between samples.
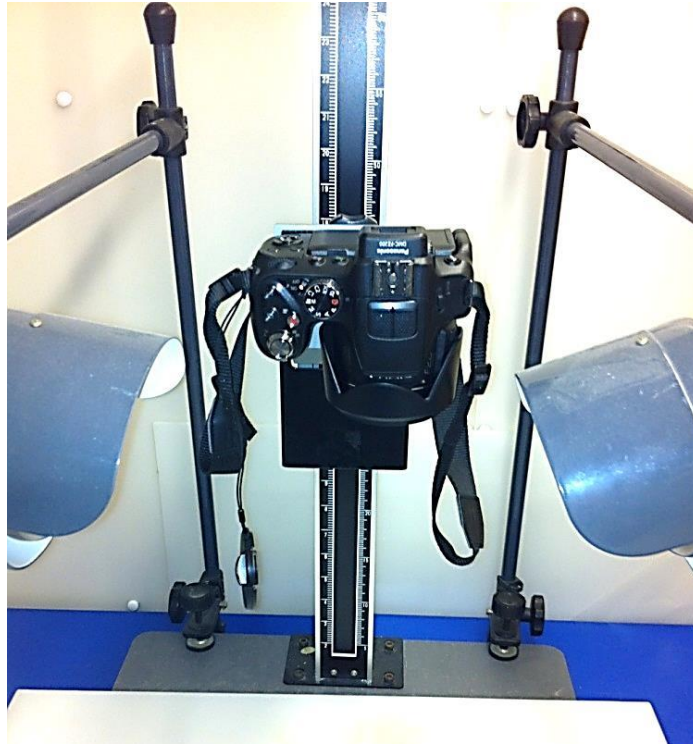
Figure 2.7 Equipment used for standardisation of light/ distance and image capture.

2.2.6.3.3 Image analysis

RAW images were imported into Corel PaintShop Pro X4 where a layered grid was applied (Figure. 2.8, overleaf). The colour dropper tool was selected and 50 points were randomly selected from the caudal section of each fish (six fish per treatment), for a total of 330 points sampled. Acetate film was used to ensure that sample points were taken from approximately the same location in each photo sample to avoid potential introduction of variation. For each of the 50 points, red, green and blue (R G B) arbitrary intensity values were then recorded for further statistical analysis.

Figure 2.8 Example RAW image of fish from the 15% algal inclusion cohort analysed using Corel PaintShop Pro X4 with grid pattern superimposed for pixel analysis.

2.2.7 Statistical analysis

All statistical tests were performed using Statgraphics Plus 4.0 (Rockville, Maryland, USA) software. All results were expressed as mean ± standard deviation (SD). Data with parametric distribution were analysed using a one-way analysis of variance (ANOVA) and significant differences between treatments were determined using Tukey's multiple comparison test ($p < 0.05$). Data with nonparametric distribution were analysed using the Kruskal–Wallis test, and significant differences were determined using Box and Whisker plots. Any count data presented as a percentage were arcsine (x 1/2) transformed to ensure normal distribution.

2.3 Results

2.3.1 Growth Performance and feed utilization

All of the diets were readily accepted by the fish. Qualitative, subjective feeding observations revealed that the diets with microalgae inclusion induced a more vigorous feeding response during first introduction of the experimental pellet feed, suggesting a possible palatability preference to microalgae inclusive diets. All tilapia were in excellent condition at the end of the trial, displaying uniform conformation, with complete dorsal and caudal fin morphology.

After completion of the 63 day trial, no significant difference between any of the growth performance values for final weight gain, SGR, FCR or final length were observed ($p > 0.05$; see Table 2.4, overleaf). The initial weight per fish ranged from 14.16 ± 0.21 g to 14.51 ± 0.12 g and had increased fourfold from the initial weight to a range of 60.83 ± 1.10 g to 64.47 ± 3.00 g final weight at the end of the trial, with no significant differences between any of the treatment groups ($p = 0.155$).

There were no significant differences in liver weight, hepatosomatic index (HSI) and condition factor (K) between any of the dietary groups ($p > 0.05$). Survival rates indicated a no significant difference between groups ($p = 0.632$). However, the 10% inclusion diet had a slightly lower survival rate due to possible hierarchy anomaly within one of the replicate tanks.

Table 2.4 Summary of growth performance, feed utilization and biological indices data.

	Control	5%	10%	15%	p Value
Initial weight (g fish^{-1})	14.37 ± 0.05	14.32 ± 0.14	14.51 ± 0.12	14.16 ± 0.21	0.092
Final weight (g fish^{-1})	64.47 ± 3.00	60.83 ± 1.10	63.67 ± 0.28	61.03 ± 2.74	0.155
SGR (% d^{-1})	2.19 ± 0.10	2.07 ± 0.11	2.08 ± 0.14	2.14 ± 0.09	0.584
FCR	1.10 ± 0.09	1.13 ± 0.09	1.13 ± 0.02	1.22 ± 0.09	0.320
Final Length (cm)*	15.01 ± 0.54	14.74 ± 0.38	15.00 ± 0.43	14.70 ± 0.45	0.873
Total feed intake (g fish^{-1})	55.27 ± 0.49	55.05 ± 1.49	55.03 ± 0.81	53.48 ± 1.49	0.297
Liver weight (g)*	1.79 ± 0.40	1.36 ± 0.15	1.49 ± 0.23	1.25 ± 0.16	0.133
HSI % *	2.65 ± 0.10	2.28 ± 0.28	2.20 ± 0.33	2.03 ± 0.53	0.135
K*	1.87 ± 0.10	1.86 ± 0.11	1.92 ± 0.15	1.81 ± 0.10	0.511
Survival (%)	93.33	90.67	90.00	92.00	0.632

(*n=3*, per treatment, unless otherwise indicated * *n* = 9)

Data represent the means ± SD

p value set at p > 0.05

SGR=Specific Growth Rate; FCR=Feed Conversion Ratio; HSI=Hepatosomatic index; K=Condition factor

2.3.2 Carcass composition

Analysis of proximate carcass composition is presented in Table 2.5. No significant differences were observed between dietary groups for moisture (ranging from 73.74 ± 1.05%to 74.18 ± 0.42%) and ash (ranging from 3.32 ± 0.21% to 3.51 ± 0.08%) at the end of the trial ($p > 0.05$). However, there was a significant difference in protein levels between the control group (16.95 ± 0.59%) and both the fish fed 10% inclusion and 15% inclusion levels (16.23 ± 0.12 and 16.20 ± 0.07, respectively; $p < 0.05$). Lipid deposition was significantly different between the control group (8.34 ± 0.07 %) and the other three groups ($p < 0.05$), with the lowest value observed in the 15% group (6.81 ± 0.04 %), followed by the 10% group at 7.40 ± 0.19%. The 5% group showed no significant difference (7.15 ± 0.14%) when compared to the 10% and 15% groups.

Table 2.5 Summary of carcass chemical composition of *O. niloticus* (% wet weight basis) for both the beginning and the end of the experiment.

	Initial (*n=4*)	Control	5%	10%	15%	*p Value*
Moisture	73.57±0.30	74.08±0.75	73.85±0.41	73.74±1.05	74.18±0.42	*0.871*
Crude protein	15.20±0.49	16.95±0.53[a]	16.33±0.25[ab]	16.21±0.23[b]	16.20±0.16[b]	*<0.05*
Crude lipid	7.64±0.28	8.34±0.27[a]	7.15±0.29[bc]	7.40±0.49[b]	6.81±0.28[c]	*<0.05*
Crude ash	4.00±0.12	3.32±0.23	3.51±0.10	3.40±0.35	3.37±0.14	*0.736*

(*n=3*, unless otherwise indicated)
Data represent the means ± SD
*[a−c] Treatments that share the same superscript letter in the same row are not significantly different ($p > 0.05$).

2.3.3 Haematological parameters

Haematological data associated with samples obtained at the end of the trial are displayed in Table 2.6. Haematocrit levels ranged 34.89 ± 0.69 % to 37.78 ± 0.51 %, haemoglobin levels ranged 6.05 ± 0.62 g dl^{-1} to 6.63 ± 0.62 g dl^{-1} and total RBC counts ranging 1.63 ± 0.74 10^6 /μm^3 to 1.88 ± 0.38 10^6 /μm^3. These parameters were not significantly affected by dietary treatment (p > 0.05). There was a significant difference in total WBC numbers between the 5% inclusion (25.73 x 10^3 cells μl^{-1}) and 15% (36.26 x 10^3 cells μl^{-1}) diets (p = 0.05). With regards to differential cell counts, the proportion of granulocytes increased from 1.5 ± 0.61 % in the control group to 3.3 ± 0.78 % in the 15% dietary treatment group (p < 0.05). However, neither the monocyte nor thrombocyte proportions were significantly affected (p > 0.05), and there were no significant differences observed with regards to serum lysozyme activity (p > 0.05).

Table 2.6 Haematological parameters of *Oreochromis niloticus* after 63 days feeding on experimental diets.

	Control	5%	10%	15%	*p* Value
Haematocrit (% PCV)	36.89 ± 2.14	37.78 ± 0.51	36.11 ± 0.69	34.89 ± 0.69	*0.809*
Haemoglobin (g dl^{-1})	6.05 ± 0.62	6.15 ± 0.82	6.63 ± 0.29	6.23 ± 0.57	*0.545*
RBC (10^6 /μm^3)	1.63 ± 0.74	1.88 ± 0.38	1.81 ± 0.11	1.86 ± 0.25	*0.073*
WBC (10^3 /μm^3)	25.73 ± 13.88 [ab]	22.35 ± 10.21 [a]	28.69 ± 11.74 [ab]	36.26 ± 15.51 [b]	*< 0.05*
Thrombocytes (%)	0.39 ± 0.38	0.44 ± 0.35	0.50 ± 0.29	0.67 ± 0.44	*0.517*
Monocytes (%)	1.11 ± 0.25	0.83 ± 0.17	1.17 ± 0.33	1.17 ± 0.29	*0.389*
Granulocytes (%)	1.50 ± 0.61 [a]	1.83 ± 0.66 [a]	1.72 ± 0.75 [a]	3.39 ± 0.78 [b]	*< 0.05*
Lysozyme (U ml^{-1})	356.31 ± 38.34	360.34 ± 78.73	312.16 ± 136.75	315.48 ± 99.27	*0.699*

Data represent the means ± SD (*n=9*)
* [a-b] Treatments that share the same superscript in the same row are not significantly different at *p* > 0.05.
PVC = Packed cell volume; RBC = Red blood cell count; WBC = White blood cell/ leukocyte count

2.3.4 Histological and SEM analysis

Morphological overviews of the mid intestinal tract and liver sections at gross and ultrastructure levels with corresponding data analysis are presented in Figures 2.9 to 2.15. All groups showed excellent morphological uniformity and structure, in line with that expected of healthy fish.

2.3.4.1 Mid-intestine histology

Representative LM images of H & E-stained transverse sections of mid intestinal tract samples are shown in Figure 2.9. Image analysis revealed that the mucosal fold (MF) length was significantly higher in the fish fed the 15% diet (24.85 ± 2.79 µm) than the control fish (20.37 ± 2.92 µm) ($p < 0.05$); see Figure 2.10). In the case of the mucosal perimeter ratio (Figure 2.11), an upward trend from 3.43 ± 0.92 AU in the control group to 3.93 ± 0.63 AU in the fish fed the highest algal inclusion level was observed, yet there were no significant differences ($p > 0.05$).
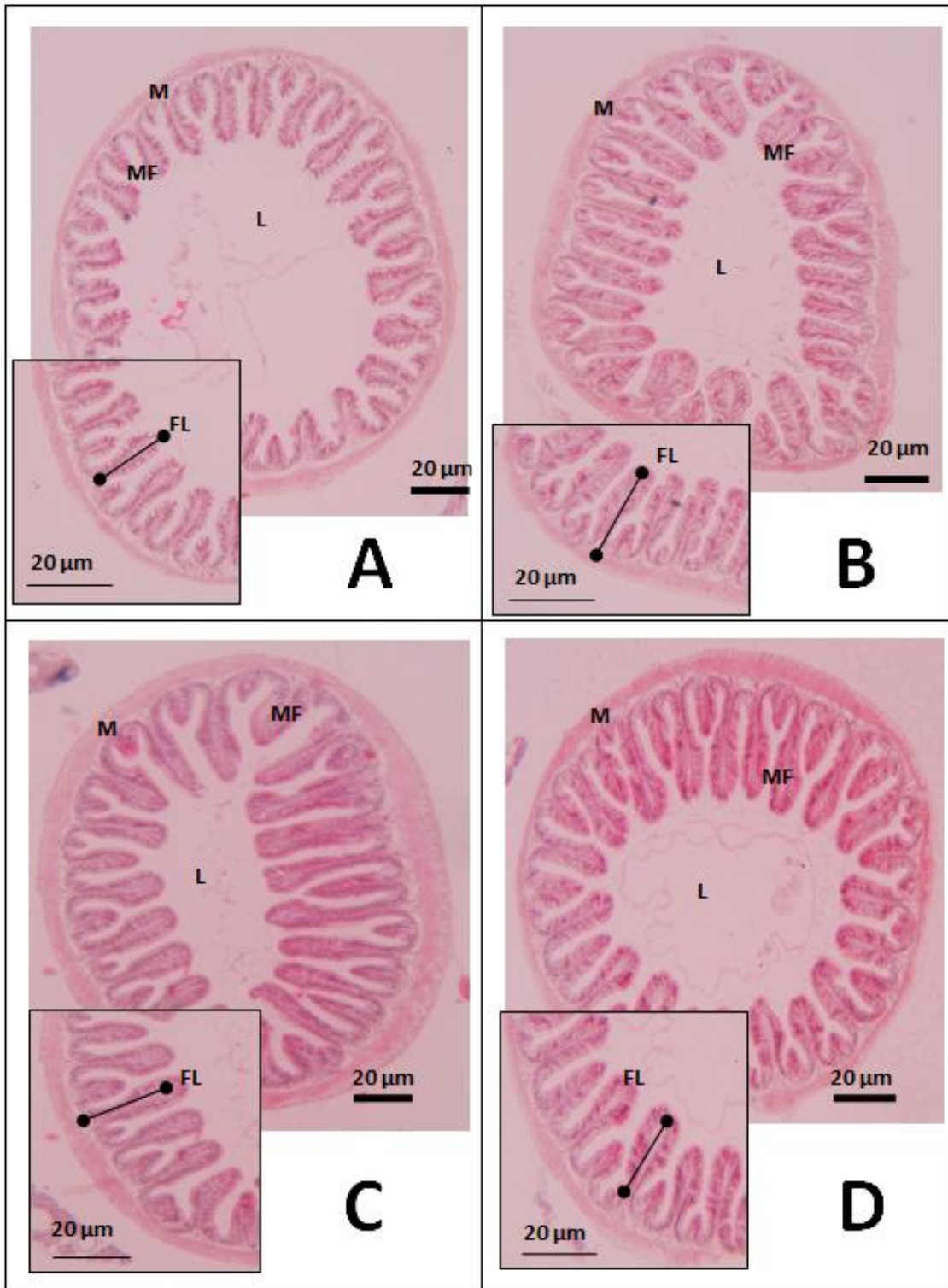
Figure 2.9 Representative 63 day LM images of H & E-stained transverse sections (5 μm) of *O. niloticus* mid-intestine samples analysed for perimeter ratio and mucosal fold length (inset). x 40 optical magnification.

(A) Control diet   (B) 5% algae inclusion (C) 10% algae inclusion and (D) 15% algae inclusion.

M: Muscularis, MF: Mucosal Fold, L: Lumen, FL: Mucosal Fold Length. Scale bar: 20 μm.
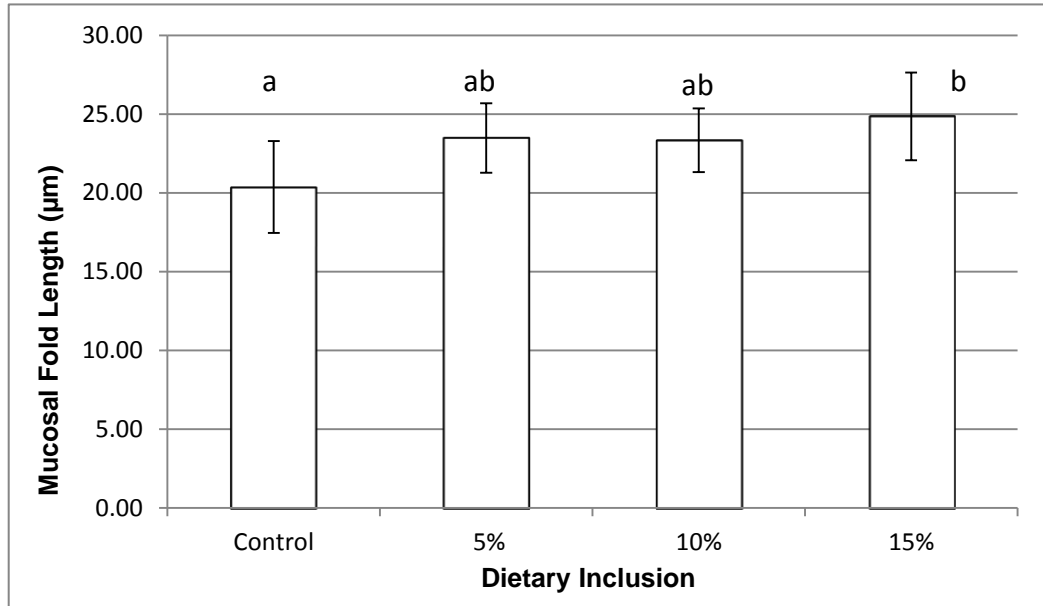
Figure 2.10 Effect of *Nannochloropsis* dietary inclusion level on mucosal fold length in *O. niloticus*.
Treatments that share a same superscript letter are not significantly different.
Error bars = Data represent the means ± SD ($p < 0.05$). ($n=6$)
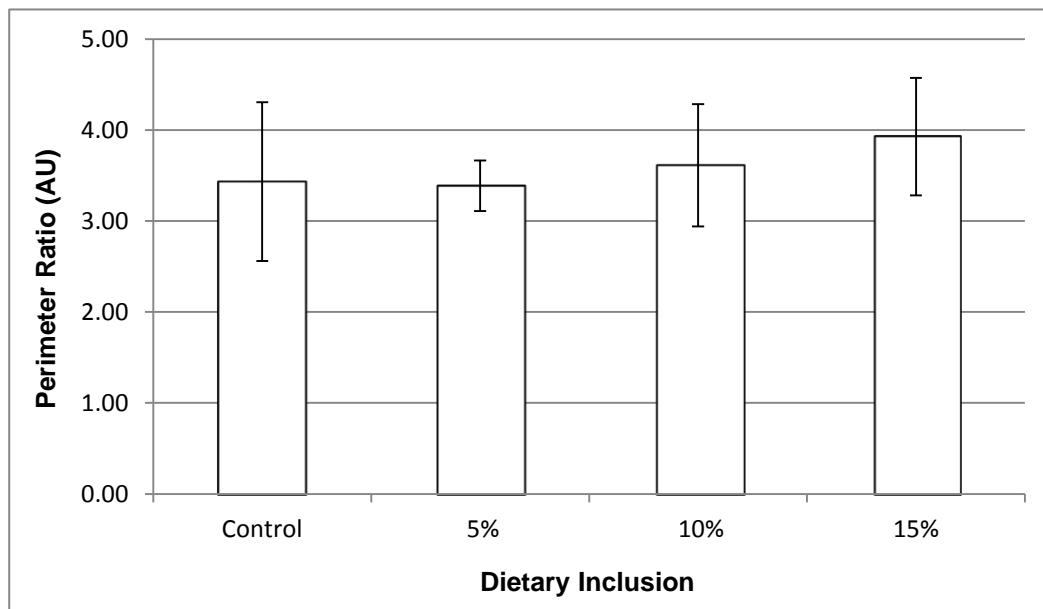
Figure 2.11 Effect of *Nannochloropsis* dietary inclusion level gut perimeter ratio in *O. niloticus.*
Error bars = Data represent the means ± SD ($p > 0.05$). ($n=6$)

2.3.4.2 Scanning electron microscopy

SEM micrographs of mid-intestine samples are shown below in Figure 2.12. Analysis of these samples revealed that *Nannochloropsis* sp*.* supplementation had no significant effect on the microvilli density ($p > 0.05$; Figure 2.13), with an overall downward trend from the control group (113.58 ± 8.74 μm^2) to the highest inclusion group (92.03 ± 12.10 μm^2).
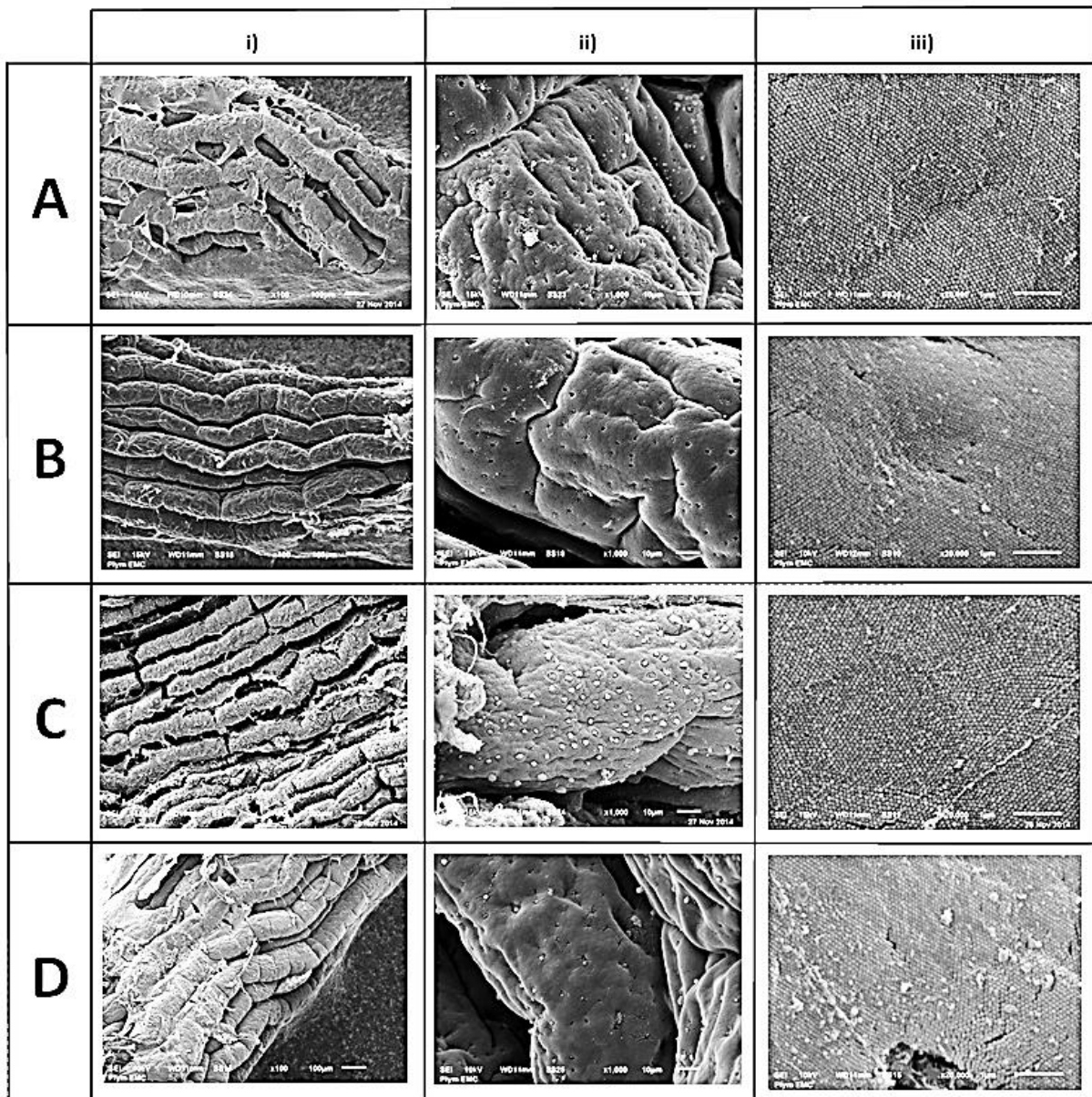
Figure 2.12 Representative scanning electron micrographs of the mid-intestine region of *O. niloticus* for each dietary treatment after 63 days.
Scale bars: i) 100μm ii) 10μm and iii) 1μm.
(A) Control diet (B) 5% algae Inclusion (C) 10% algae Inclusion and (D) 15% algae Inclusion.
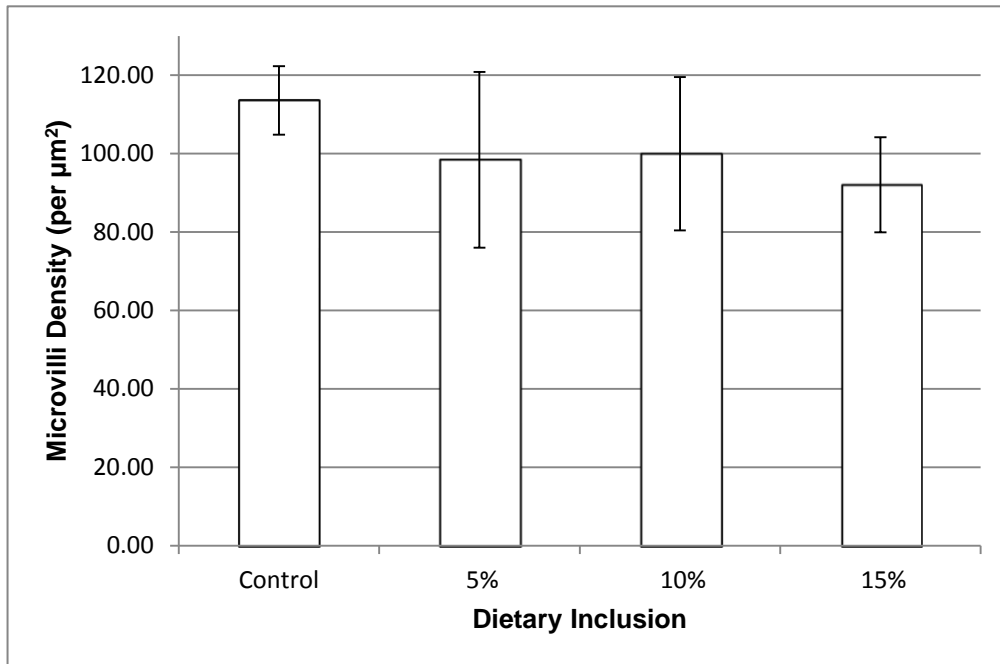
Figure 2.13 Effect of dietary inclusion level of *Nannochloropsis* on microvilli density as assessed by SEM and image analysis of mid-intestinal samples of *O. niloticus.*

Error bars = Data represent the means ± SD. ($p > 0.05$). ($n=9$).

2.3.4.3 Liver histology

Representative LM images of hepatic tissue are shown overleaf in Figure 2.14. Histological appraisal of nuclei counts from hepatic tissue showed a significant difference between the control group (19.22 ± 2.57 per 100 μm^2) and all of the experimental diet groups (25.36 ± 1.21 per 100 μm^2; $p <$ 0.05, Figure 2.15). However, there were no significant differences in the weights of liver samples taken at the end of the 63 day trial (see Table 2.3). At x 10 magnification, images appeared to show lipid-like droplets in the 15% inclusion as seen in Figure 2.14 (D i), which were not observed in any of the other dietary groups.
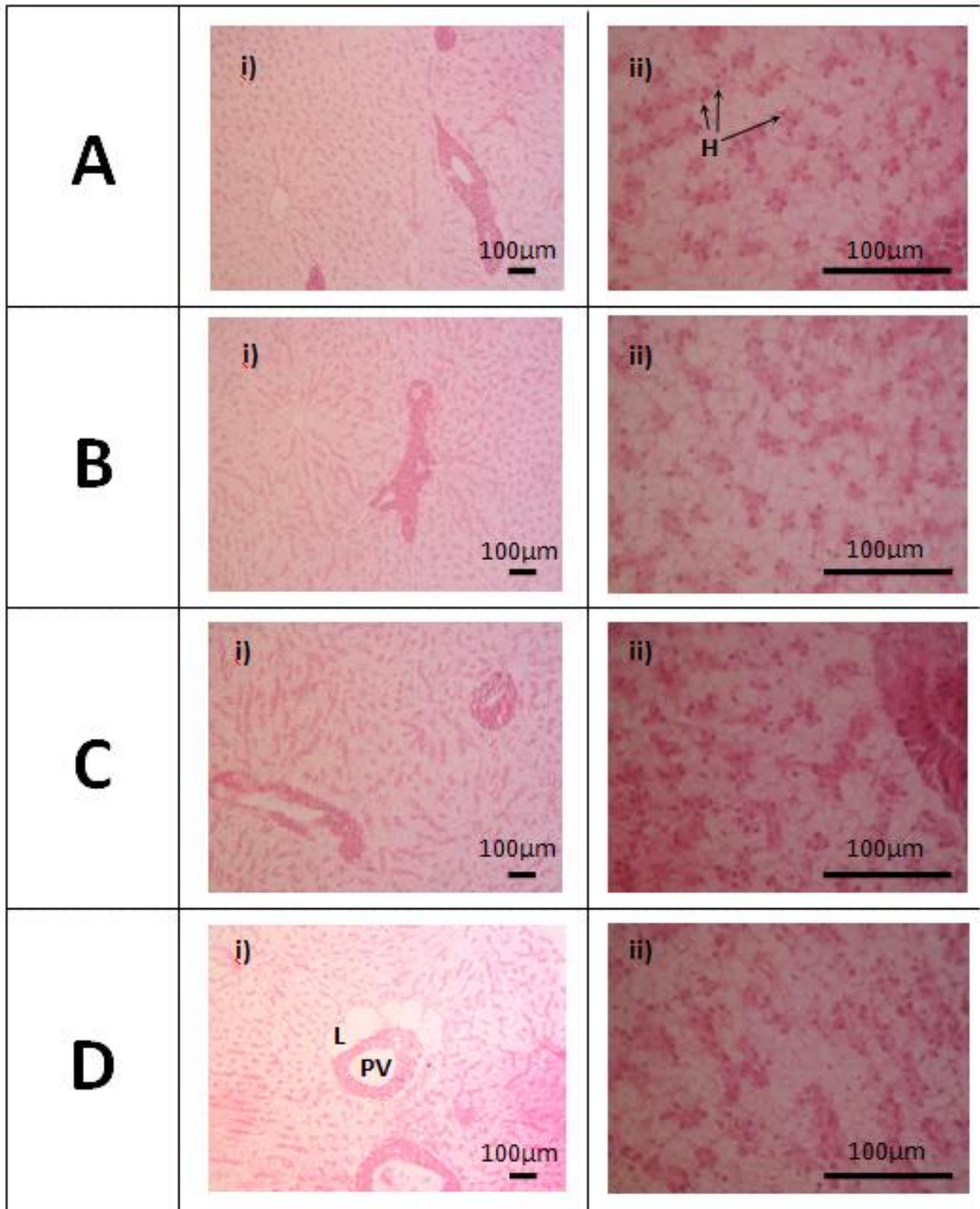
Figure 2.14 Representative LM images of stained liver sections of *O. niloticus* used for appraisal of hepatocyte nuclei counts.

Treatments: (A) Control diet   (B) 5% algae Inclusion (C) 10% algae Inclusion and (D) 15% algae Inclusion.

(i) Scale bar: 100μm for general structure at x 10 magnification and (ii) Scale bar = 100μm for image analysis at x 100 magnification.

H= hepatocytes, L= Lipid droplets, PV= portal vein.
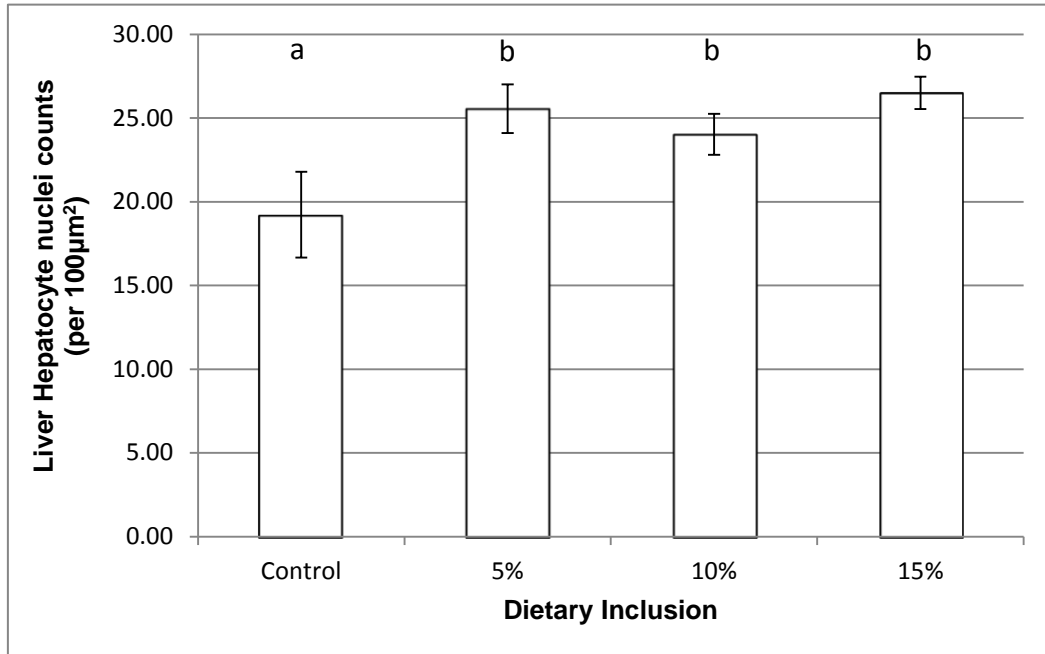
Figure 2.15 Effect of dietary inclusion level on hepatocyte nuclei density assessed using H & E-stained transverse sections (5µm) of liver samples.

Treatments that share a same superscript letter are not significantly different from each other. Data represent the means ± SD. ($p < 0.05$). (*n=6*).

2.3.5 Photographic colouration analysis

Initial visual observation of trial groups suggested a colour enhancement in the caudal and dorsal regions of fish fed the algal supplemented diets when compared with a separate group of fish fed on the control and fish fed on a commercially available pelleted feed (Figure 2.16 A, B & D) for the same time period. This was additionally confirmed by the side-by-side visual observations of the head region for the control group and the 15% inclusion group (as seen in Figure 2.16 C). Neither the mean greyscale value (as an indicator of brightness) nor the mean colour index (Figure 2.18 D) revealed any significant difference (p = 0.278) amongst all of the dietary groups, implying that efforts to standardise the photographic appraisal process were suitable as a baseline indicator.

Photographic appraisal of all dietary trial groups (Figure 2.17) indicated that there was a significant difference at the 10% - 15% inclusion level (192.73 ± 12.24 AU and 194.04 ± 10.66 AU, respectively) compared to the control / 5% inclusion groups (177.54 ± 8.59 AU and 173.90 ± 12.66 AU respectively, $p < 0.05$; Figure 2.18 A). This was additionally reflected in the red index values (the proportion of this colour to the whole) with the same significance trend observed (Figure 2.18 A).

The variation of mean colour and index values for green and blue (Figure 2.18 B & C) showed somewhat opposing trends when compared to red mean colour and index values. A decreasing mean green colour was found in the control group (127.33 ± 22.35) and the 15% inclusion group (108.91 ± 27.80) with a significant difference ($p < 0.05$) observed between the control and all other dietary inclusion groups. Green index values showed a significant difference ($p < 0.05$) between the control group (0.309 ± 0.02) and both higher inclusion levels of 10% and 15% (0.275 ± 0.03) and a significant difference ($p < 0.05$) between the middle groups of 5% (0.293 ± 0.03) and 10% inclusion (0.277 ± 0.03). The mean blue colour

showed the same trend as the mean green colour with a significant difference ($p < 0.05$) between the control group (105.09 ± 26.76) and the two higher inclusion groups of 10% and 15% (82.62 ± 29.13 and 84.55 ± 27.18 respectively). The blue index was significantly different ($p < 0.05$) between the control and 5% inclusion groups (0.25 ± 0.04) and the higher inclusion diets of 10% and 15% (0.21 ± 0.04).
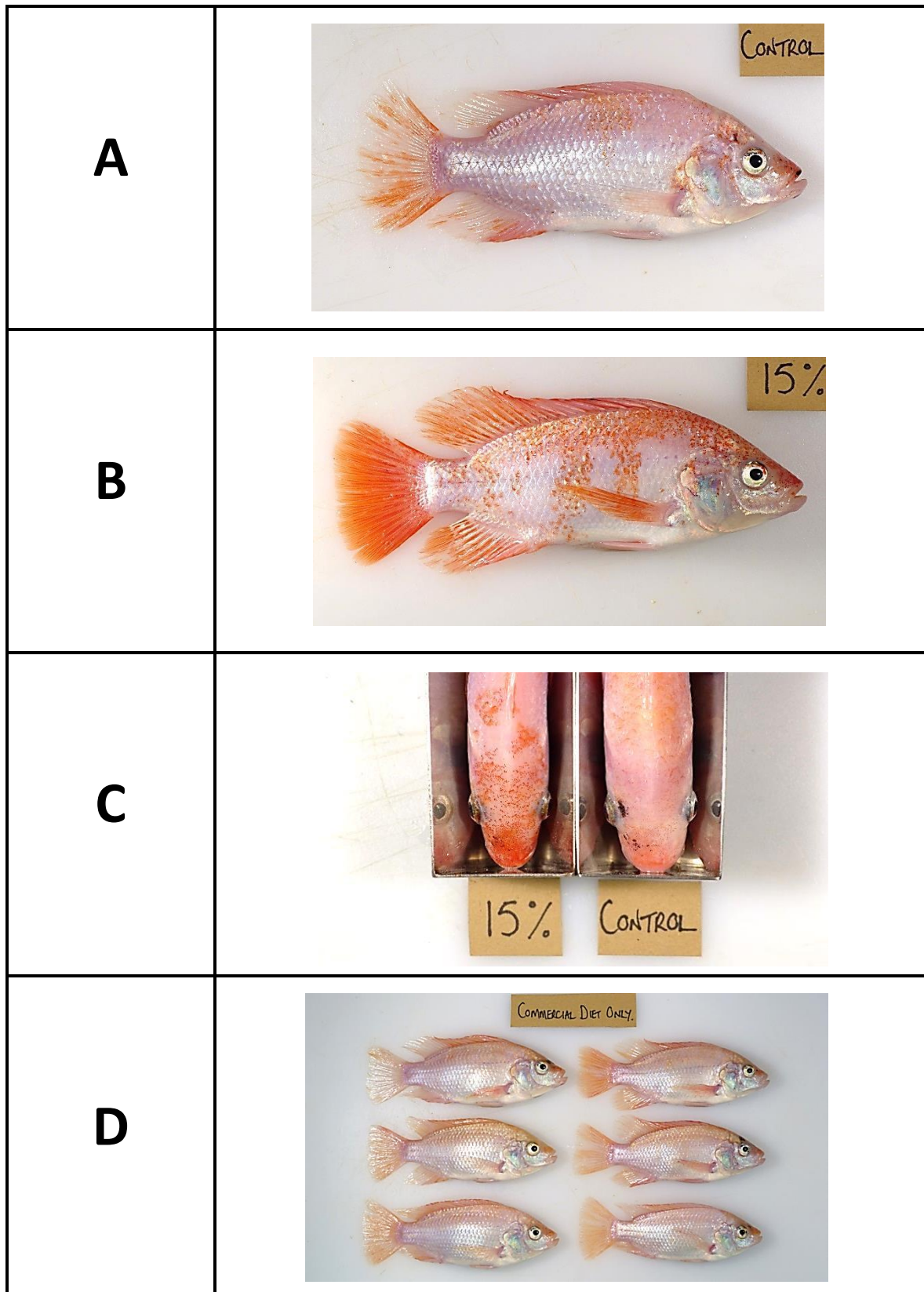
Figure 2.16 Comparative images of *O. niloticus,* gathered at the conclusion of nutrition trial.
(A) Control diet, (B) 15% algae Inclusion (C) Control & 15% algae inclusion - head area from above,
and (D) fish fed a commercial feed (EWOS), for the same time period of nutrition trial.
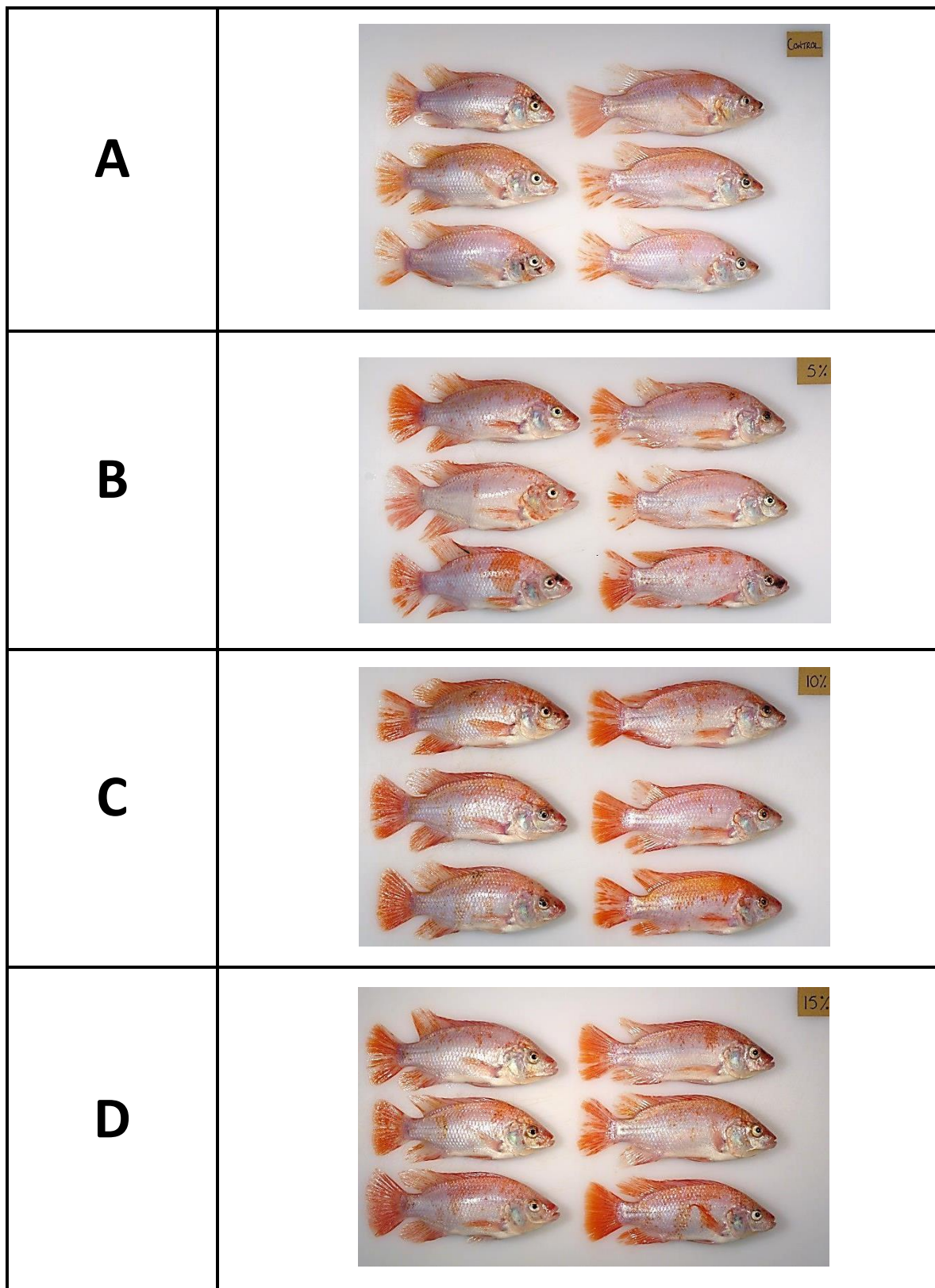
Figure 2.17 Comparative images of *O. niloticus* colouration differences after 68 days of feeding on the experimental diets.
 (A) Control diet,   (B) 5% algae Inclusion (C) 10% algae Inclusion, (D) 15% algae Inclusion.
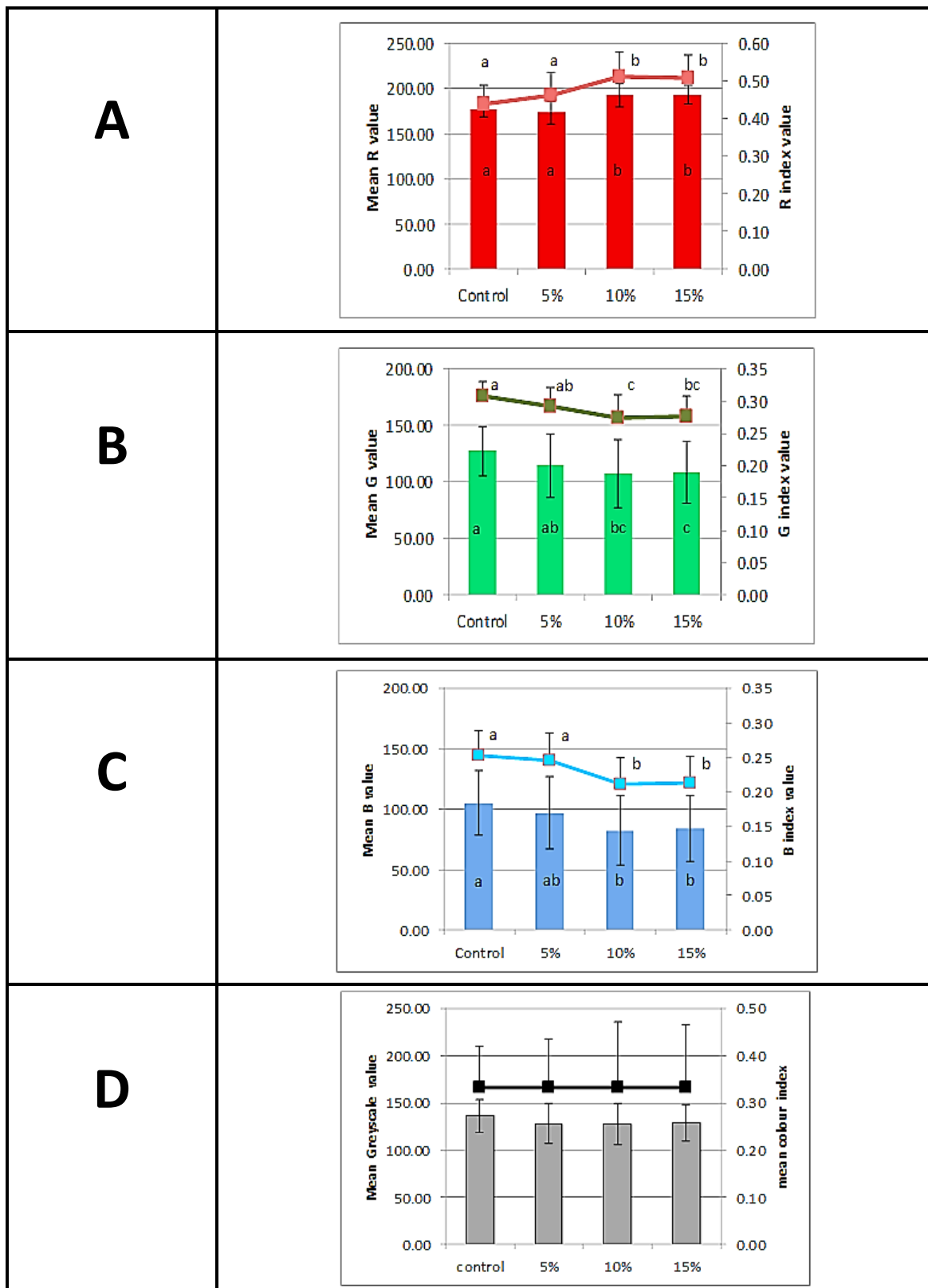
Figure 2.18 Results of photographic appraisal of colour enhancement in *O. niloticus* in response to increasing dietary inclusion of *Nannochloropsis* after 68 days.

Results are presented as mean colour indices (lines) and mean absolute values for each colour (bars).

Treatment groups that share the superscript letter are not significantly different.

Data represent the means ± Error bars represent SD ($p < 0.05$) ($n=6$).

2.4 Discussion

Nannochloropsis is considered to be one of the most interesting phytoplankton species in marine biology, as it represents a source of a variety of high value, natural products that have several important biotechnological applications (Borowitzka, 2013). Over the past decade, *Nannochloropsis* spp. have primarily been utilised in the aquaculture industry as a source of natural carotenoids, proteins and polyunsaturated fatty acids (Camacho-Rodriguez *et al.*, 2013)**.** *Nannochloropsis* spp. have been investigated extensively as a raw material for biodiesel production, due to their naturally high lipid content (Spolaore *et al.*, 2006) and through innovative technologies the sustainable production of these second generation biofuels on a commercial scale has increased greatly, offering considerable opportunities for the future (Gouveia and Oliveira, 2009).

To the author's knowledge, no other tilapia nutrition trial to date has been published on the dietary incorporation of dried *Nannochloropsis* spp.. This present study is therefore believed to be the first to investigate a commercially viable, defatted and dried proprietary *Nannochloropsis* meal in diets for a very important commercial aquaculture species (Nile tilapia, *Oreochromis niloticus*). Overall growth performance compared well with all three balanced microalgae incorporated diets, when compared with the formulated control diet in this study. The formulated diets contained slightly higher levels of protein than those described by Jauncey (1998b) for this species, with all four iso-nitrogenous % of the diet delivered at mean 36.58% crude protein, with all four also formulated to be iso-energetic diets for gross energy supplied at mean 19.61 MJ kg $^{-1}$.

When incorporating a *Nannochloropsis* sp. and *Isochrysis* sp. mix at 0%, 5% and 30% replacement of fish meal in diets for juvenile Atlantic cod (*Gadus morhua*), Walker and

Berlinsky (2011) reported a deterioration of growth, concluding that this was due to a reduction in palatability with increased algae inclusion levels for what is predominantly a carnivorous species; this was not observed in this trial when tilapia consumed diets to the fixed feeding level of 3% bw/day $^{-1}$. Another carnivorous species was recently investigated by Skrede *et al.* (2011), who utilised three microalgae species, *Nannochloropsis oceanica*, *Phaeodactylum tricornutum* and *Isochrysis galbana* in a digestibility trial with mink (*Mustela lutreola*), a model used for salmon and other farmed mono-gastric species. Microalgae sources were included at graded levels up to 24 % in the feed. The authors revealed a linear regression in apparent crude protein digestibility and although the algae contributed a minor proportion of dietary lipids, lipid digestibility declined with increasing inclusion of algae. The algae used had been freeze-dried prior to the trial and the authors hypothesised that the cell walls of some of the utilised algal species may have been more easily broken down by digestive processes than others. This differed to a study by El-Dahhar *et al.* (2014), who investigated the use of lab-based starter cultures of *Nannochloropsis oculata* in grey mullet (*Liza ramada*) for larval diets testing growth performance and feed utilization. Their results showed that the highest weight gain when compared to the control was recorded with the 21% inclusion followed by 28% inclusion, which was also reflected in the reported FCR values.

During the diet formulation process of the current study, fishmeal was incorporated at 200 g kg^{-1} consistently through all diets, owing to a number of studies that have reported, replacement with high levels of plant-based proteins without any animal protein is detrimental to growth and health of fish, unless supplemented with essential amino acids such as methionine and lysine (El-Sayed, 1999; El-Saidy and Saad, 2011; Gonzalez-Felix *et al.*, 2011; Thompson *et al.*, 2012). The general consensus is that *Oreochromis* require 5% to 12%

lipid in formulated diets (Lim *et al.*, 2011) with 5% being the minimal requirement (Chou and Shiau, 1996). A number of studies have reported the same relationship in formulation practices (Jauncey, 1982; Dabrowski *et al.*, 2010b; Hussein *et al.*, 2013), but these studies all had increasing protein levels as their investigative objective. Desilva *et al.*, (1991) experimented with differing protein levels (15%, 20% and 30%) with a series of lipid inclusions (6%, 18%, 24% and 30%) for each treatment. At all three protein levels the best growth was obtained with diets of 18% lipid, possibly indicating the protein-sparing capabilities of young red tilapia in this study. The protein-sparing capability increased with increasing dietary lipid content up to 18% and decreased thereafter.

In the present investigation there appears to be a discrepancy with the dietary lipid levels. Despite being iso-caloric in terms of gross energy content, oil levels varied and were trending higher in diets with elevated algae content. This may be due to the analytical method employed and it is suggested that lipid extraction is more efficient from algae due to high surface area and uniformity of cells. It should be noted that the algae retained ~ 6% residual oil, although specified as an oil extracted by-product of *Nannochloropsis* whole cells. Tilapia have been reported to utilize vegetable oil that is high in omega 6 (n-6) fatty acids better than fish oil that is rich in omega 3 (n-3) fatty acids for maximum growth efficiency (Lim *et al.*, 2011). This study utilised corn oil as a good source of (n-6) polyunsaturated lipid, nevertheless Perez Ribeiro *et al.*, (2008) reported that tilapia showed poor utilization of corn oil when compared with olive oil and soybean oil. The results of carcass proximate composition on termination of the feeding trial imply that although the recommended dietary lipid was incorporated (> 5%) with uniform NFE (available carbohydrate) delivered throughout the respective diets, body crude lipid deposition decreased with algae inclusion, with a similar trend for crude protein. This may have resulted in the prioritised utilisation of

all lipid and protein for maintenance and growth and as a consequence resulting in a reduction of nutrient accumulation. It should be noted however, that the control diet was formulated with selected quality ingredients representing a typical commercial diet for tilapia with superior specifications for comparison with the other dietary formulations containing the test algal product. Another possible explanation for a lowered body lipid level may be due to the faster transit of algae cells within the gastrointestinal tract of the tilapia. Further, it was observed in the trial that water within the fish holding system was noticeably cloudy after feeding; suggesting that in the high algae groups' tilapia excreted more biomass. Further experiments must be undertaken to validate this hypothesis of a reduction in algal digestibility with increasing dietary algae incorporation.

With respect to the measured health parameters and haematological indices no significant differences were observed in haematocrit, haemoglobin or RBC counts; the levels for these parameters were within the reported normal range according to (Hrubec *et al.*, 2000), who studied haematology and plasma chemistry reference intervals for the same species. Relative to the control group, there were no significant differences in WBC levels. However, in terms of the proportional composition of WBC subpopulations, tilapia fed the highest algal inclusion displayed higher granulocyte levels, the results still falling into the range suggested by Hrubec *et al.* (2000) at 21.55 – 154.60 WBC (10^3 /μm^3).

It should also be noted that microscopically identifying cell types with subtle differences is challenging and highly subjective, which is why, for instance, the larger groups of leukocytes were chosen and not granulocyte sub-groups for instance.

Lysozyme is an important component of the innate immune system of teleosts as it is the first line of defence against bacterial invasion. Saurabh and Sahoo (2008) investigated lysozyme activity in a number of fish species, including in the plasma of *Oreochromis*

niloticus, and reported that it varies depending on fish sex, age and size, as well as the season, water temperature, pH, toxicants, infection levels and degree of stressors. In the present study, lysozyme activity ranged between 315 and 356 units (time and absorbance) ml^{-1} with no significant differences observed between experimental groups. Gao *et al.*, (2012) suggested that in tilapia, lysozyme is expressed at a high level in the intestine, indicating perhaps that it might have both digestive and defensive functions that may coincidentally aid in the digestion of the cell walls of microalgae. A study conducted by Gerken *et al.* (2013) indicated that the permeability of *Nannochloropsis* cell walls was increased by treatment with the enzyme, lysozyme. This may account for the downward trend observed in the current study of lysozyme activity with algal inclusion.

The intestine is a specialized tubular structure, with the mid gastro-intestinal region encompassing the greater surface area, manifested by elongated mucosal folds. Each of these "finger like" projected folds is composed of villi that form the mucosa (a multitude of further projections to further increase the surface area) of the epithelial membrane, these are densely packed with microvilli making available a greater surface area for nutrient absorption (Merrifield *et al.*, 2011). One of the objectives of this study was to assess this area for morphological changes that may arise from microalgae inclusion in the experimental diets. The histological features of the tilapia *Oreochromis niloticus* digestive system have been investigated over the course of several decades (Kayanja *et al.*, 1975; Osman and Caceci, 1991; Smith *et al.*, 2000), although differences in qualitative and quantitative outcomes are evident. This investigation followed a more recent study by Merrifield *et al.* (2011) that examined both gross- and ultrastructure of mid-intestinal morphology of *Oreochromis niloticus*. Analysis of LM in the current study revealed no signs of histological damage and the epithelium conformed to healthy morphology as observed in

the control. Mucosal fold lengths were in the range of 20-25 μm across the treatment groups, with a significant difference occurring between the control and higher inclusion of microalgae, inferring that mucosal folds were longer at this level.

The gut perimeter/internal mucosal fold ratio takes into consideration the fish size, a higher perimeter ratio indicating an increased internal absorptive surface area brought about by more numerous or longer mucosal folds. As was evaluated in this study, the 15% algal inclusion led to significantly longer mucosal fold length, which in turn led to higher PR. This may be beneficial as an increase in absorptive area may potentiate or allow for greater nutrient absorption. This study was directly comparable to the findings of Merrifield *et al.* (2011), with all dietary groups yielding PR values of 3 -4 AU. The findings of this study imply that microalgae inclusion level could have effects on fast growing tilapia and that this may have implications on the growth performance of these fish. Although, high inclusion rates of some singular plant proteins appear to have a negative effect on intestinal morphology and physiology of a wide variety of finfish (Drew *et al.*, 2007), the nutritive value of diets will depend on the digestibility of any individual ingredient (Sklan *et al.*, 2004). The increase in mucosal fold length may also have been a result of compensation, for the assimilation of algal cells. As potential interactions among ingredients resulting in gut morphology changes are possible, any further studies should demonstrate these interactions to identify potential changes in digestion efficiency.

Scanning electron microscopy (SEM) analysis of the microvilli density had a declining trend from the control group with no significant differences observed, although microvilli did not change, the length of the microvilli was not measured. Evidently more work is required to elucidate the efficacy of microalgae products on gut morphology, integrity and function in further studies with this species. The effects on larger fish will be of interest to fish

producers and feed manufacturers offering lower cost diets or in terms of utilizing novel products even at modest inclusion levels such as unicellular algae, bacterial proteins and yeasts amongst single cell protein sources.

Interesting findings were also obtained from the assessment of liver histological profiles. Liver tissue sections showed clear and normal tilapia morphological confirmation across all dietary inclusions when compared to the control group. No pathological lesions or necrosis of any cells were observed. A higher hepatocyte nuclei count per 100 μm^2 was recorded with significant differences observed between all algal incorporated diets when compared with the control group, implying possible hyperplasia of cells, leading to denser cell volume. Liver density is higher; this may be reducing nutrient availability and assimilation, suggesting that fewer nutrients may have been available from the continuously changing organ. Internal changes occur quicker than external changes and this trial was a "snap shot" in time and a longer trial may have facilitated a better understanding of this rapidly changeable and complex organ associated with nutrient status. Also, it should be noted that a possible pseudo-effect of the apparently increased cell numbers could be due to a more overall density in that field of view along the narrower hepatic sinusoid channels between hepatocyte rows in cross sections examined.

The methods applied in the current study for colour evaluation presented results with regard to the effects on the epithelial skin colouration of *O. niloticus* when fed formulated diets with inclusion of *Nannochloropsis* meal containing carotenoids (canthaxanthin and astaxanthin). Over the past few decades, scientists have been researching microalgae strains and species for other alternative uses, such as natural alternatives to replace the use of synthetic carotenoids. Production of synthetic carotenoids has imposed a high cost for many

years, as marketing strategies for farmed salmon have favoured a red colour that fits consumers' preferred image of salmon flesh. To achieve this, synthetic carotenoids have been added to fish feed, so that farmed salmon ingest it in the same way as wild salmon (Torrissen and Christiansen, 1995). Gouveia *et al.* (2003) carried out a study with two trials to investigate skin colour enhancement in ornamental species, koi carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), by feeding a dietary carotenoid supplement of freshwater microalgae biomass (*Chlorella vulgaris*, *Haematococcus pluvialis* and also the cyanobacterium *Arthrospira maxima* (Spirulina)) in a comparative study with synthetic astaxanthin. The authors concluded that a more active red colouring was achieved in skin colour with the diets containing algae biomass, not the synthetic carotenoid, in both trials. The analysis of skin colour assessment was performed by reflectance spectroscopy for hue and saturation using a portable Minolta Chroma Meter CR-300.

The experimental approach employed here was adapted from a study carried out by Stevens *et al.* (2007), in which the authors aimed to set out step-by-step guidelines for the use of digital photography, to obtain accurate data in animal colouration studies. The adapted information from their study proved to be an excellent solution for low cost, non-disruptive exploration into the preliminary carotenoid uptake in skin of *O. niloticus*. The current study showed a significant increase in the Red (R) value component and decreases in the Green (G) and Blue (B) value components of the RGB system, resulting in a more profound visual red colour, in the caudal fin sections of all the algae supplemented diets when compared to the control fish. This was in agreement with Kouba *et al.*, (2013) who showed that when feeding microalgae biomass containing the carotenoid astaxanthin to angelfish (*Pterophyllum scalare*) for 28 days, the red component of the skin colour increased and the green and blue components decreased with the inclusion of spray-dried cells of the

green microalgae *Haematococcus pluvialis* and whilst a visual red colour increased, no significant influence of microalgae addition was detected on the final live weight of the fish. Similar results were reported in experiments with other species of aquaculture importance, where vivid skin colouration for large yellow croaker (*Larimichthys croceus*) (Yi *et al.*, 2015), flowerhorn cichlid (*Amphilophus citrinellus*) (Sornsupharp *et al.*, 2015), red porgy (*Pagrus pagrus*) (Kalinowski *et al.*, 2007) and to a lesser extent red Nile tilapia (Gomes *et al.*, 2012) are all appreciated by consumers, who subconsciously relate colour to nutritive value, healthiness and high quality products that leads to higher market values being achieved (Koller *et al.*, 2014). In certain Asian communities red tilapia resemblance to red snapper or red sea bream gains a premium price and may also obtain a premium as it is the colour of "good luck". Natural carotenoid inclusion-based diets possess huge potential for the aquaculture industry for prolonging shelf life via antioxidants and skin coloration impacts, and the latter requires further investigation.

2.5 Conclusions

The results from the current study indicate that a low level (up to 15%) dietary inclusion of a proprietary *Nannochloropsis* sp. by-product meal did not cause any significant adverse effects on growth performance indices and overall health of *Oreochromis niloticus* at the end of the 63 day feeding trial. This implies that the algal biomass composed of cells was sufficient for digestion and utilisation by tilapia, a most important fish of economic value. The algae was capable of supplying key nutrients such as protein, amino acids and potentially important lipids that may confer beneficial attributes to human health.

The health indices measured revealed only elevated granulocyte populations of significance in the 15% algae inclusion group when compared with the control dietary group, however, the results conformed to haematological reference values for this species, although it would be beneficial to investigate this further to ensure correct and definitive leucocyte subgroups are identified.

Further work should include a more comprehensive immunological evaluation for better understanding of colonization interactive dynamics of the gut microbiota of tilapia fed microalgae in relation to immune competence. Future research or studies are also required to elucidate liver conformation in novel ingredient nutritional trails for comparisons to be accomplished as available information is sparse.

It is well documented that carotenoids are found in plants, algae and photosynthetic bacteria. However, the consequence of carotenoid uptake and subsequent colouration development of red Nile tilapia has not, to the author's knowledge, been reported or investigated before in such detail. The findings in this study imply that very modest contributions of this proprietary *Nannochloropsis* possessed a significant red colour

enhancement for this species, which could have a huge impact on not only consumer acceptability and interest for an already abundant aquaculture species, but also for the wider ornamental fish trade and aquarium community as a whole.

Although it is understood that the present price of microalgae biomass continues to make inclusion of microalgae on a large commercial scale economically non-viable, as the Earth's resources, such as fossil fuel, deplete and technology improves then biomass production of this valuable resource for biofuel will become increasingly competitive.

Chapter 3

Assessment of commercial proprietary *Chlorella* sp. in diets for *Oreochromis niloticus*

3.1 Introduction

The genus *Chlorella* belongs to the class Trebouxiophyceae, which were first described by Beijerinck (1890) as predominantly a freshwater unicellular spherical or ellipsoid celled organism. This group has undergone many subsequent taxonomic revisions based on morphology and phylogenetic sequencing (Luo *et al.*, 2010). Bock *et al.* (2011) reported that the genus is a heterogeneous assemblage of species with urgent need for a revision, with this genus presently consisting of five true *Chlorella* species: *Chlorella vulgaris, C. lobophoa, C. sorokiniana, C. heliozoae* and *C. variabilis,* based upon biochemical and molecular assessment.

Beijerinck (1890) described *Chlorella vulgaris* as a spherical, eukaryotic, unicellular alga containing chlorophyll-*a* and chlorophyll-*b* as its photosynthetic pigments that are contained within the chloroplast, with a cell diameter of approximately 2 – 10 µm. The cell wall contains hemicelluloses, which accounts for the stability and rigidity of the cells. It has an asexual reproductive cycle, with optimal conditions one mature cell can divide into four new cells every 16 – 20 hours. The algal cells utilize sunlight for photosynthesis with a single chloroplast, surrounded by starch grains (Juneja *et al.*, 2013).

Chlorella spp. are of high economic importance due to their chemical composition; they also possess a wide range of essential nutrients, such as proteins (Becker, 2007b) , carotenoids (Del Campo *et al.*, 2007) and polysaccharides (Juneja *et al.*, 2013). The crude protein content holds particular potential for animal feed or human consumption, and varies from about 15 % to 88 % of dry matter (Guccione *et al.*, 2014). The cellular content is dependent on the algal species and strain selection, cultivation methods and procedure, harvesting time, growth medium composition, lighting and production stressors (Becker, 2007b). Digestibility issues

exist with the resilience of the cell wall and the consequent difficulty with which the proteins and lipids can be extracted; this can interfere with nutrient absorption, or conversely be a useful binding agent in forming of feed pellets (Slocombe *et al.*, 2013).

During the growth phase, some species of microalgae accumulate high levels of carotenoids, providing a major natural source of diverse pigments as a part of their biomass and represent interesting biological alternatives to traditional sources (Del Campo *et al.*, 2007) β-carotene is a pigment increasingly in demand and has a wide variety of market applications: as a food colouring agent (the most important outlet); as pro-vitamin A (retinol) in food and animal feed; as an additive to cosmetics and multi-vitamin preparations and as a health food product (Estevez *et al.*, 2001; Miranda *et al.*, 2001).

The aims of this trial were to analyse a proprietary hybrid strain of *Chlorella* sp. supplied by a commercial source. A control diet was formulated as a reference for the study and three diets with varying levels of microalgal inclusion. The first inclusion level of 15% was formulated for direct comparison with highest inclusion used in Trial 1 (reported in the previous chapter), with 30% and 60% inclusion levels formulated for scientific assessment to fully realise the upper constraints on growth performance, health indices and potential morphological changes that may occur in tilapia fed this algal source. Given the observed effects on integument pigmentation found in trial 1, similar characteristics on skin colour were evaluated in this second investigation.

3.2 Materials & Methods

3.2.1 Description of microalgae and production

A 6 kg sample of hybrid *Chlorella* spp. was acquired from a commercial source, although the exact combination of *Chlorella* species used to produce the sample strain was not revealed due to commercial confidentiality. The growth conditions are also not fully disclosed for the same reason. In brief, this strain was cultured via indoor proprietary enclosed lab-based photo-bioreactors (PBR) until sufficient mass was accumulated. Cultures were then transferred to outdoor culturing processes that were sustained by use of recirculating media, requiring only the addition of inorganic nutrients and carbon dioxide. The biomass was harvested, free of chemicals or flocculants; dewatered via centrifuge until 5-10% moisture remained, producing algal sludge. Lipid components of the algal cell were removed via a patented method and then the residue dried, before it was packaged and sent for analysis at the Plymouth University Aquaculture Health and Nutrition unit. Scanning electron microscopy screening was carried out to explore ultrastructure of the microalgae supplied, as described in section 2.2.6.5.2 for observations of cell integrity and structure. Figure 3.1 shows rounded structures that appear mostly intact, or at least swollen, with not all cells "cracked", although no comparison is available to compare the extent of fragmentation of cell walls. Proximate compositional analysis was conducted using wet chemistry methods as described in section 2.4.5 using standard (AOAC, 2012) protocols (Table 3.1).
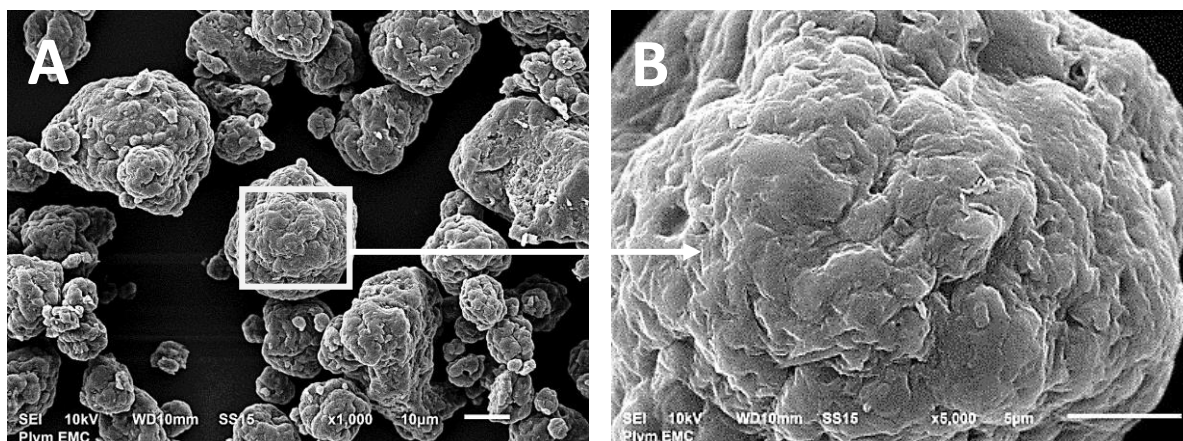
Figure 3.1 Scanning electron microscope micrographs of algal sample showing cell cluster structure and integrity.

Several cells appear intact - highlight insert (B).

A) Scale bar = 10 µm and B) Scale bar = 5 µm.

Table 3.1 Proximate composition of *Chlorella* spp. algae (dry matter basis).

Composition	%
Protein	46.86
Ash	10.78
Lipid	1.63
NFE + fibre	40.73
Gross energy	19.35 (MJ kg^{-1})

NFE = (Nitrogen-free extracts)

Moisture [i]   7.89

[i] Values are given based on a dry matter basis

3.2.2 Feed formulation & diet preparation

Experimental diets were prepared in 5 kg batches. Four iso-nitrogenous and iso-energetic diets were formulated to meet the known nutritional requirements of tilapia according to guidelines (Table 3.2) (Jauncey, 1998b) utilising a standard feed formulation software package (Feedsoft Professional®).

Table 3.2 Dietary formulation & proximate composition of experimental diets.

Ingredients g kg^{-1}	Control	15%	30%	60%
Herring Meal [a] (LT94)	150.0	150.0	150.0	150.0
Fish Oil [b]	35.0	35.0	35.0	35.0
Soybean Oil [c]	32.5	22.2	26.9	38.8
Soybean [e] (hamlet HP100)	150.0	-	-	-
Lysamine Pea Protein [f]	277.2	263.6	172.1	32.9
Corn Starch [d]	330.3	354.2	291.0	118.3
Chlorella [g]	-	150.0	300.0	600.0
Vitamin-mineral premix [h]	20.0	20.0	20.0	20.0
CMC - Binder [i]	5.0	5.0	5.0	5.0
Total (g)	1000	1000	1000	1000
Proximate Composition (% dry matter basis)				
Dry matter	6.59	6.34	4.55	6.82
Crude protein (N x6.25) [j]	38.71	38.19	39.60	37.79
Lipid [j]	9.81	8.16	9.98	10.23
Ash [j]	5.28	4.98	6.98	9.49
NFE [k]	39.61	39.67	38.89	35.67
Gross energy (MJ kg $^{-1}$)	19.77	19.47	20.64	20.25

1 [a] CC Moore, Dorset, UK
2 [b] BioMar Group, Scotland
3 [c] Soybean Oil, Local Oriental Store, UK
4 [d] Sigma-AldrichLtd, UK
5 [e] Hamlet Protein, Denmark
6 [f] Roquette Frères, France
7 [g] Heliae, Arizona, USA

8 [h] Premier nutrition vitamin/mineral
9 premix
10 [i] CMC (Carboxymethyl cellulose) Sigma-
11 Aldrich Ltd., UK
12 [j] Values are given based on a dry matter
13 basis
14 [k] NFE (Nitrogen-free extracts) = dry
15 matter – (crude protein + crude lipid + ash)

16

17 Dry milled ingredients were weighed into a new 5 litre plastic container and mixed

18 uniformly to ensure homogenous distribution of the diet components and then the dry-

19 blended ingredients were mixed for approximately 30 minutes in a Hobart food mixer

20 (Hobart Food Equipment, Australia). Fish oil and Soybean oil were then added separately

21 and gradually, in a continuous flow. After further mixing, warm water was added to the

22 mixture to form a consistency suitable for cold press extrusion (La Monferrina P6, La

23 Monferrina, Asti, Italy) to produce 4 mm strands.

24 The resulting strands were carefully laid out into cleaned polypropylene trays, lined with tin

25 foil. These trays were subsequently transferred to a warm air oven (Genlab, MINO/200/SS/F,

26 Cheshire, UK) and were left to dry at 40$^{o C}$ for 48 - 52 hours, turning approximately every 12

27 hours. Diets were cooled and crushed to a suitable size for juvenile tilapia (Figure 3.2),

28 placed into new 5 litre plastic containers that had been previously sterilized with 70% IMS,

29 labelled and kept in a dark refrigerated environment (5 °C) until use in this trial.

30

31

32 **Figure 3.2** Pelleted feed, produced in the Feed Production Centre, Aquaculture Health and Nutrition
33 Unit, Plymouth University.

3.2.3 System set up and experimental design

A group of 500 monosex male (XY genotype) Nile tilapia (*Oreochromis niloticus*) were imported from Fishgen, UK (www.Fishgen.com), into the Aquaculture, Health and Nutrition Research Facility at Plymouth University, UK. An acclimation period of 6 weeks followed to take the individual weight of the newly arrived fish from 0.05 g to 15 g approximate start weight. During this period, feeding was carried out 8 - 10 times daily to approximately 30% body weight of the total biomass (according to Feeding Tables published in Jauncey (1998b)), using a fine grade commercial pellet (EWOS Micro 015P – 5 P). Suitable tilapia were carefully selected from the main imported group, to achieve an overall distribution of 35 fish per tank. All twelve of the experimental tanks of fish weighed within 3% of each other, with a total of 420 fish used at the start of the trial. The experimental fibreglass tanks had a capacity of 80 litres, utilising standard mains freshwater supply in a closed recirculation system (RAS; see Figure 2.4). The RAS used a 3 kW inline heater (Electro Titanium Aquatic heater) to deliver temperature controlled water (27°C ± 0.5 °C) and was also fitted with a commercial U.V steriliser unit (TMC, P2, 55W lamps). This provided 35 fish per tank for each of the four diets, and three tanks per dietary treatment. Each tank of fish was fed a 3% body weight ration; three times daily at the same time each day, with half rations on Sunday, prior to biweekly weighing. The trial was conducted for a period of 35 days, with sampling conducted on the final day.

3.2.4 Growth and feed utilization calculations

Weight Gain (WG), Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), Hepatosomatic

Index (HSI), Condition factor (K) and Survival were all assessed as described in section 2.2.4

3.2.5 Sampling

Prior to commencement of the nutritional trial, samples of all diets and ingredients were

collected for chemical analysis. Twelve fish were randomly sampled at the start of

experiment for carcass composition analysis, prior to distribution as described in section

2.3.3. At the end of the experiment, 3 fish per tank ($n=9$ per treatment) were removed

randomly from each tank, euthanized with buffered MS222 (tricaine methanesulphonate,

Pharmaq) at 200 mg L^{-1} (with sodium bicarbonate at 400 mg L^{-1} for pH buffering) and

individually weighed for carcass composition analysis. Fish sampled for haematology

parameters were anaesthetised in compliance with the Home Office Animal Scientific

Procedures act (1986).

3.2.5.1 Haematology

Haematological assays were carried out, 3 fish per tank ($n=9$ per treatment) as described in

section 2.2.6.1.

3.2.5.2 Histology

Histological appraisal of the mid-intestine (light microscopy and scanning electron

microscopy) and liver (light microscopy) was conducted on three fish per tank ($n=9$) as

described in section 2.2.6.2.

3.2.5.3 Photographic assessment of skin colouration

Photographic assessment of skin colouration was undertaken on two fish per tank (*n=6).*
The procedure for image capture is described in section 2.2.6.3.2 (pp 78).The procedure for image analysis of captured RAW images is described in 2.2.6.3.3 (pp 80).

3.2.6 Statistical analysis

Results are expressed as mean ± standard deviation. Data with parametric distribution were analysed using a one-way analysis of variance (ANOVA) and the significant differences between treatments ($p < 0.05$) determined using Tukey's multiple comparison test. Data with nonparametric distribution were analysed using Kruskal-Wallis tests, and significant differences were determined using Box and Whisker Plot graphs. Any data presented as a percentage were arcsine (x 1/2) transformed to ensure normal distribution. All statistical tests were performed using Statgraphics Plus 4.0 (Rockville, Maryland, USA) software.

3.3 Results

3.3.1 Growth performance and feed utilization

All groups of Nile tilapia readily accepted experimental diets, without obvious palatability limitations. Feeding the highest inclusion diet of 60% *Chlorella* turned the system water green after the 1st day of feeding, and thereafter for the duration of the trial. Although 3% body weight of daily feed intake was achieved throughout the trial, extended time was required within this 60% inclusion group as the trial progressed. It should be noted that no water stability tests were conducted on the pelleted feed to confirm algal leeching during feeding.

The trial commenced with no significant difference in weight of tilapia between any of the 12 experimental tanks (22.68 ± 0.18 g, Table 3.3). After 5 weeks, the control group had effectively tripled in weight with a final mean weight of 64.64 ± 1.83 g. Compared to the control, all other treatment groups showed a significant difference in final weight and overall gained less weight ($p < 0.05$). Fish fed the 60% inclusion diet displayed significantly lower final mean weight and weight gain than all other groups (final weight 38.24 ± 1.25 g; weight gain of 15.42 ± 1.18 g, $p < 0.05$). Final mean length measurements reflected these weight differences with a similar reduction with higher algal inclusion rates when compared to the control group, with the length of fish fed 60% inclusion significantly shorter than the control and 15% inclusion groups ($p = 0.05$).

The Specific Growth Rate (SGR) data revealed a similar trend, with a significant difference between the 60% group of 1.31 ± 0.31 % per day and all the other groups (control = 2.98 ± 0.20, 15% treatment = 2.90 ± 0.16 and 30% treatment = 2.44 ± 0.05) ($p < 0.05$). The FCR was also significantly affected ($p < 0.05$). Selected biological health indices were significantly

affected by varying inclusion levels, for example, HSI indices in the 15% and 30% inclusion

treatments were significantly higher than the 60% inclusion treatment ($p < 0.05$). Whole

liver weights were significantly lower in the 60% group at 0.59 ± 0.21 g compared to the

control ($p < 0.05$), no differences were observed between the other treatment groups.

Similarly, the condition (K) factor of fish at the end of the trial showed a significant decline

between the control and 15% groups and the 60% group ($p < 0.05$). No significant

differences in survival were observed ($p = 0.266$).

Table 3.3 Growth performance, feed utilization and biological indices of *O. niloticus*

	Control	15%	30%	60%	*p* value
Initial weight (g fish^{-1})	22.57 ± 0.35	22.70 ± 0.27	22.63 ± 0.05	22.82 ± 0.07	*0.0734*
Final weight (g fish^{-1})	64.64 ± 1.83 [a]	55.79 ± 2.44 [b]	51.77 ± 2.99 [b]	38.24 ± 1.25 [c]	*<0.05*
SGR (% d^{-1})	3.07 ± 0.42 [a]	2.83 ± 0.36 [a]	2.47 ± 0.18 [a]	1.32 ± 0.17 [b]	*<0.05*
FCR	0.99 ± 0.27 [a]	1.04 ± 0.44 [a]	1.23 ± 0.56 [a]	2.31 ± 0.18 [b]	*0.02*
Final Length (cm) *	14.42 ± 1.10 [a]	14.36 ± 0.81 [a]	13.80 ± 0.80 [ab]	12.96 ± 1.06 [b]	*<0.05*
Total feed intake (g fish^{-1})	39.45 ± 1.56 [a]	36.28 ±1.31 [ab]	35.08 ±2.31 [ab]	34.12 ±1.54 [b]	*<0.05*
Liver weight (g) *	1.31 ± 0.31 [a]	1.43 ± 0.33 [a]	1.30 ± 0.28 [a]	0.59 ± 0.21 [b]	*<0.05*
HSI % *	1.96 ± 0.34 [ab]	2.31 ± 0.39 [a]	2.39 ± 0.29 [a]	1.67 ± 0.36 [b]	*<0.05*
K factor *	2.22 ± 0.12 [a]	2.08 ± 0.14 [ab]	2.04 ± 0.12 [b]	1.64 ± 0.44 [b]	*<0.05*
Survival (%)	96.43	98.57	97.38	95.24	0.266

n=3, per treatment, unless otherwise indicated (* *n* = 9)

Data represent the means ± SD

*Treatments that share the same superscript letter in the same row are not significantly different ($p > 0.05$).

SGR=Specific Growth Rate; FCR=Feed Conversion Ratio; HSI=Hepatosomatic index; K=Condition factor

3.3.2 Carcass composition

The moisture content from 60% inclusion group was significantly higher in comparison to all other groups ($p < 0.05$), which showed no significant differences between one another, with all groups similar to the initial fish moisture content (Table 3.4). Crude protein content analysis revealed no significant difference between control group (15.80 ± 0.56%) and the 15% inclusion (15.40 ± 0.49%). Decreasing further with the 15% and 30% inclusion levels (15.19 ± 0.92%) showing the same non-significant difference. Fish fed the 60% inclusion diet contained significantly lower protein levels (14.37 ±0.30%) than all other groups ($p < 0.05$).

Lipid carcass content was significantly lower in the 60% group (5.14 ± 0.44%) compared to the all other groups ($p < 0.05$). In the case of the 15% group a significant increase in carcass lipid content (10.77 ± 0.71%) was observed when compared with control group (9.56 ± 0.75%)

The ash content of the 60% inclusion group (3.67 ± 0.29) was significantly higher than the other dietary groups ($p < 0.05$). There were no significant differences between the other treatment groups.

Table 3.4 Carcass composition of *O. niloticus* (*% dry weight basis*) for initial and at the end of the experiment

(%)	Initial (n=4)	Control	15%	30%	60%	*p Value*
Moisture	73.57±0.30	71.17±0.95 [a]	70.06±0.73 [a]	71.33±0.44 [a]	75.74±0.87 [b]	<0.05
Crude Protein	14.67±1.92	15.80±0.56 [a]	15.40±0.49 [ab]	15.19±0.92 [bc]	14.37±0.30 [c]	<0.05
Crude Lipid	7.64±0.28	9.56±0.75 [a]	10.77±0.71 [a]	9.42±0.53 [a]	5.14±0.44 [b]	<0.05
Crude Ash	4.00±0.13	3.07±0.07 [a]	3.10±0.14 [a]	3.17±0.14 [a]	3.67±0.29 [b]	<0.05

(*n=3*, unless otherwise indicated)

Data represent the means ± SD

*Treatments that share a same superscript in the same row are not significantly different at $p > 0.05$.

Comparisons were made between dietary treatments and excluded the initial values.

3.3.3 Haematological parameters

Haematological and immunological assessments of blood samples obtained at the end of the trial period are displayed in Table 3.5. Haematocrit (% PCV), haemoglobin, RBC and WBC levels were not affected by dietary inclusion groups ($p > 0.05$). When studying WBC subpopulation proportions, no significant differences were observed for thrombocyte or monocyte levels ($p > 0.05$). However, the granulocyte percentage was significantly increased from 2.50 ± 1.09% in the control group to 4.33 ± 0.76% in the 15% inclusion group ($p < 0.05$).

Table 3.5 Haematological parameters after 35 days of a nutritional investigation of microalgae *Chlorella* sp. in diets for *O. niloticus*.

	Control	15%	30%	60%	*p-value*
Haematocrit (% PCV)	36.28 ± 2.66	34.11 ± 3.69	37.44 ± 4.91	33.56 ± 0.51	*0.712*
Haemoglobin (g dl^{-1})	9.03 ± 0.03	7.31 ± 0.02	7.83 ± 0.00	7.87 ± 0.04	*0.743*
RBC(10^6/µm^3)	3.87 ± 1.78	4.35 ± 0.91	3.30 ± 1.15	3.13 ± 1.31	*0.057*
WBC (10^3/µm^3)	38.50 ± 19.89	43.84 ± 24.66	34.11 ± 16.30	36.64 ± 17.01	*0.498*
Thrombocytes (%)	0.83 ± 0.17	0.78 ± 0.10	0.78 ± 0.10	0.78 ± 0.10	*0.989*
Monocytes (%)	1.39 ± 0.86	2.06 ± 0.10	1.44 ± 0.25	1.89 ± 0.77	*0.071*
Granulocytes (%)	2.50 ± 1.09 [a]	4.33 ± 0.76 [b]	3.33 ± 1.01 [ab]	4.00 ± 1.83 [ab]	*<0.05*
Lysozyme (U ml^{-1})	320.40 ± 70.75	341.60 ± 52.41	304.23 ± 137.67	293.38 ± 128.69	*0.653*

(*n=9* per treatment)
*Treatments that share a same superscript in the same row are not significantly different at *p* < 0.05.
PVC = Packed cell volume; RBC = Red blood cell; WBC = White blood cell

3.3.4 Histological and SEM analysis

Histological appraisal of the mid-intestine and liver sections at gross and ultrastructure levels with corresponding data analysis are presented in Figures 3.3 – 3.9.

3.3.4.1 Mid-intestine histology

Analysing transverse sections of mid-intestine stained with haematoxylin and eosin showed no constriction of the canal lumen in any sample, with uniform pattern of the mucosal folds observed (Figure 3.3) revealing no significant differences in mucosal fold length (Figure 3.4) between any of the groups ($p > 0.05$). The perimeter ratio analysis (Figure 3.5) also revealed no significant decrease in the ratio of internal surface area to external area of mid-intestine samples of tilapia fed all diets containing microalgae when compared with the control group.
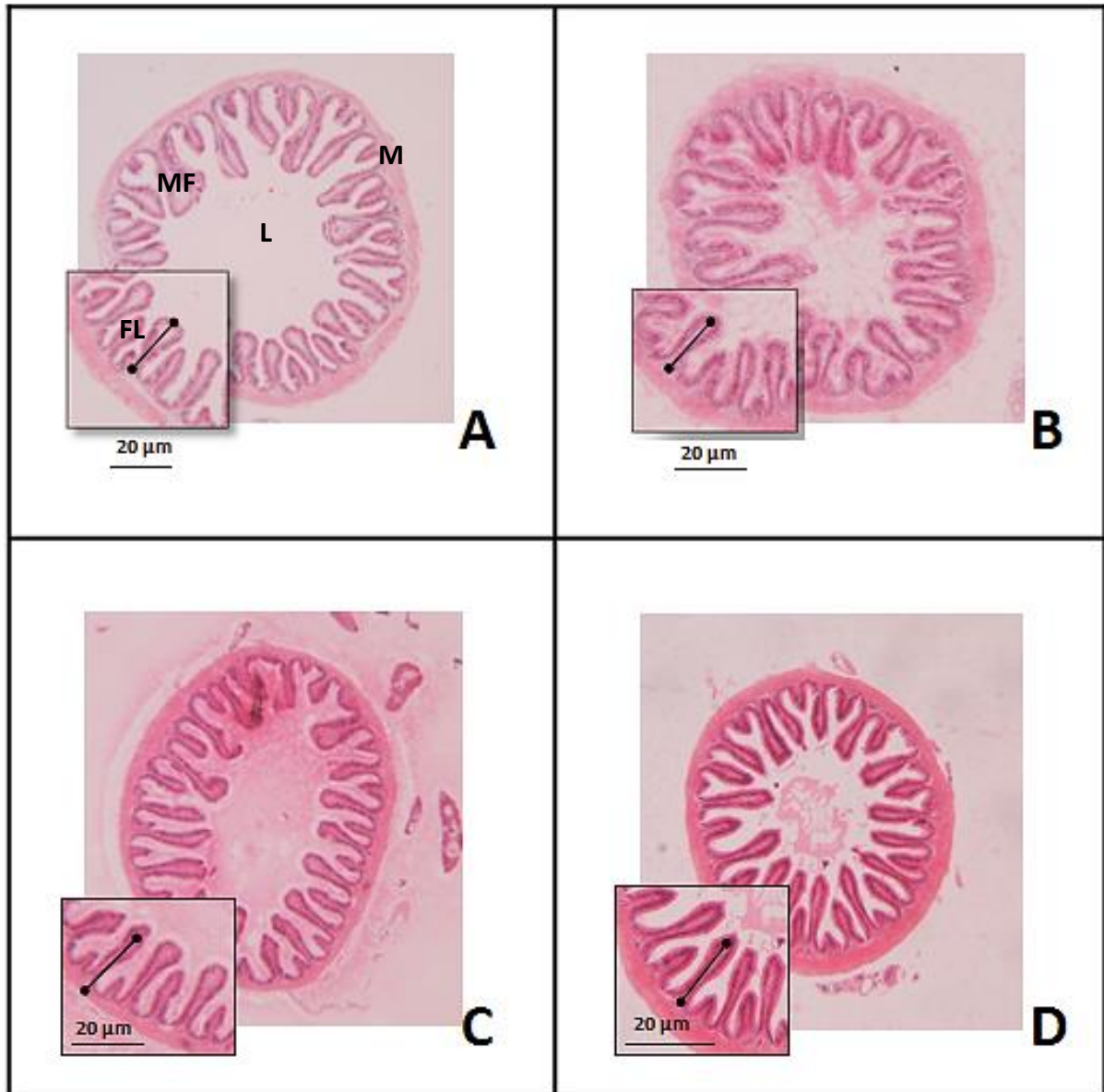
Figure 3.3 Haematoxylin and eosin (H & E) transverse sections (5µm) of *O. niloticus* mid-intestine samples analysed for perimeter ratio and mucosal fold length (inset).

Treatment groups: (A) =Control diet, (B) =15% inclusion, (C) =30% inclusion and (D) =60% inclusion. Scale bars = 20 µm. Abbreviations used, M: Muscularis, MF: Mucosal Fold, L: Lumen, FL: Fold Length.
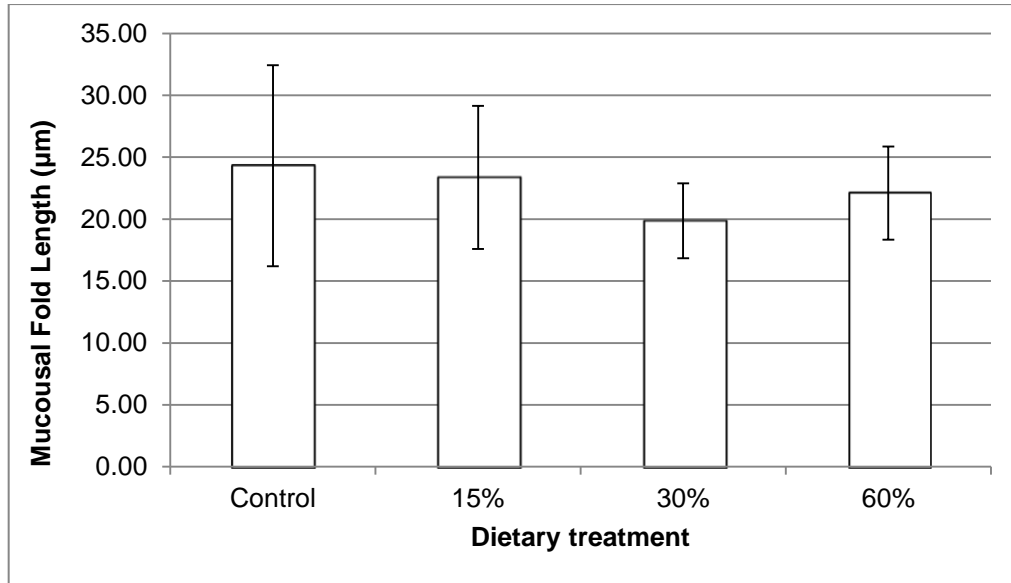
Figure 3.4 Histological appraisal of mucosal fold length from mid-intestine sample of *O. niloticus* for each diet*,* in relation to dietary inclusion levels*.*
Means ± SD (*n*=9)
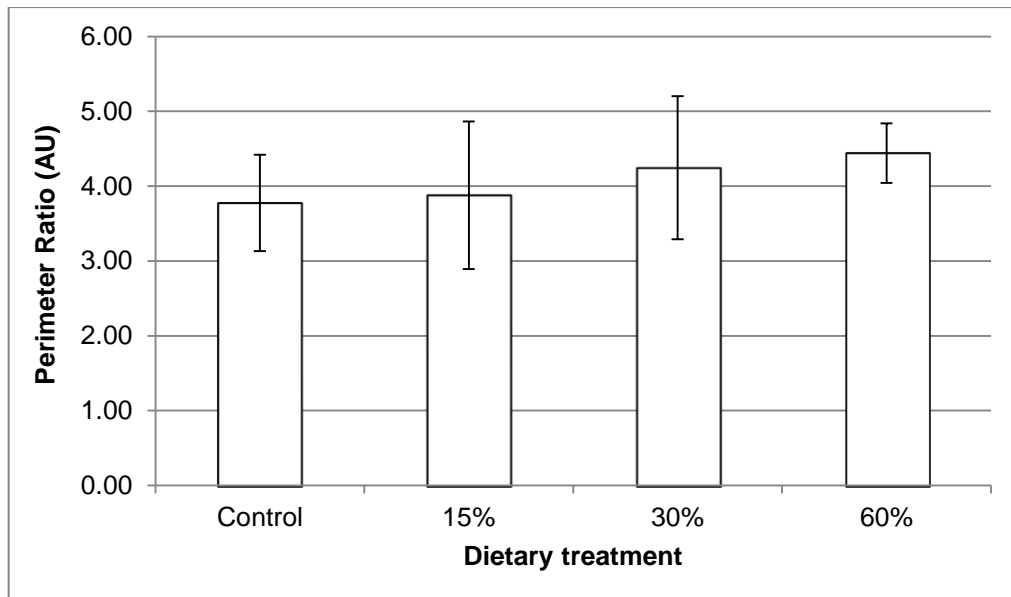
Figure 3.5 Histological appraisal of gut perimeter ratio from mid-intestine section of *O. niloticus* for each diet, in response to increasing dietary *Chlorella* inclusion levels.
Means ± SD (*n*=9)

3.3.4.2 SEM analysis

Employing SEM for appraisal of intestinal ultrastructure (Figure 3.6) revealed no significant differences in microvilli density, with an average value of 115.19 ± 2.30 μm^2 in the control group, ranging up to a maximum of 118.81 ± 1.10 μm^2 for the 30% group (Figure 3.7).
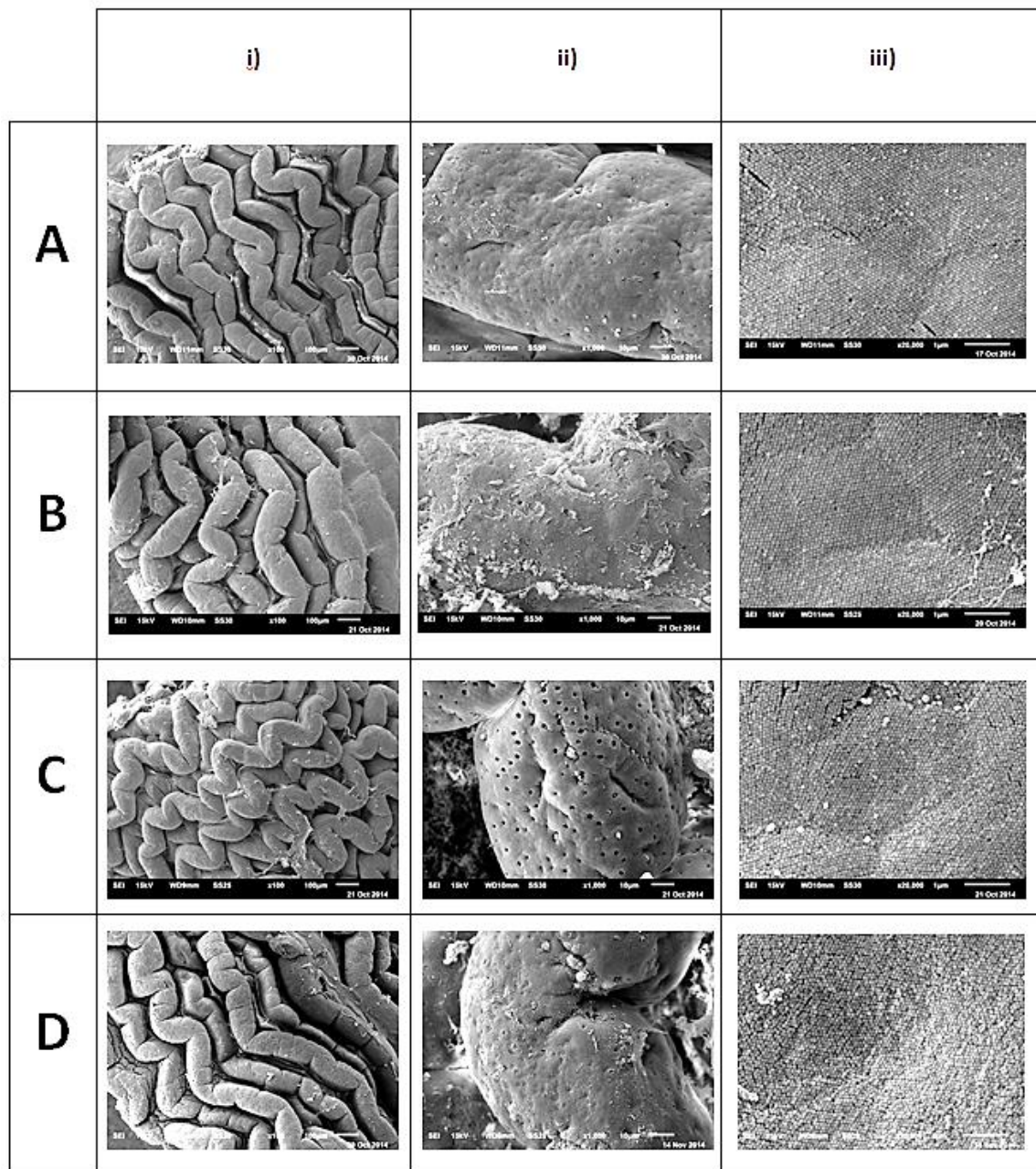
Figure 3.6 Representative scanning electron microscope images of mid-intestine region of *O. niloticus* for each dietary treatment after 35 days.

Scale bars: i) = 100 μm, ii) = 15 μm, and iii) = 1 μm.
Treatment groups: (A) = Control diet, (B) = 15% inclusion, (C) = 30% inclusion, and (D) = 60% inclusion.
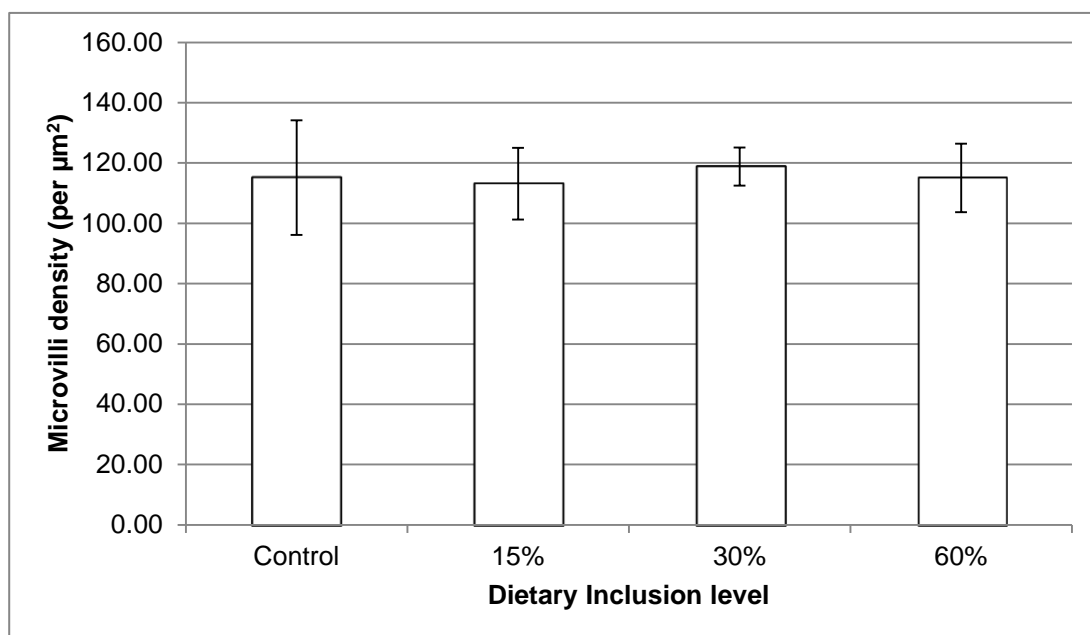
Figure 3.7 Scanning electron microscopy appraisals of microvilli density images from mid-intestinal samples of *O. niloticus*.
Means ± SD (*n*=9)

3.3.4.3 Liver histology

Histological analysis of the liver revealed normal architecture with no morphological or pathological abnormalities observed at gross level (Figure 3.8i) in any of the dietary groups; the hepatocytes appeared centrally located with appropriate spherical structure. No significant differences were observed in the hepatocyte nuclei counts between the control, 15% and 30% groups (Table 3.9). However, in the case of the 60% inclusion group, hepatocyte nuclei numbers were significantly higher ($p < 0.05$).
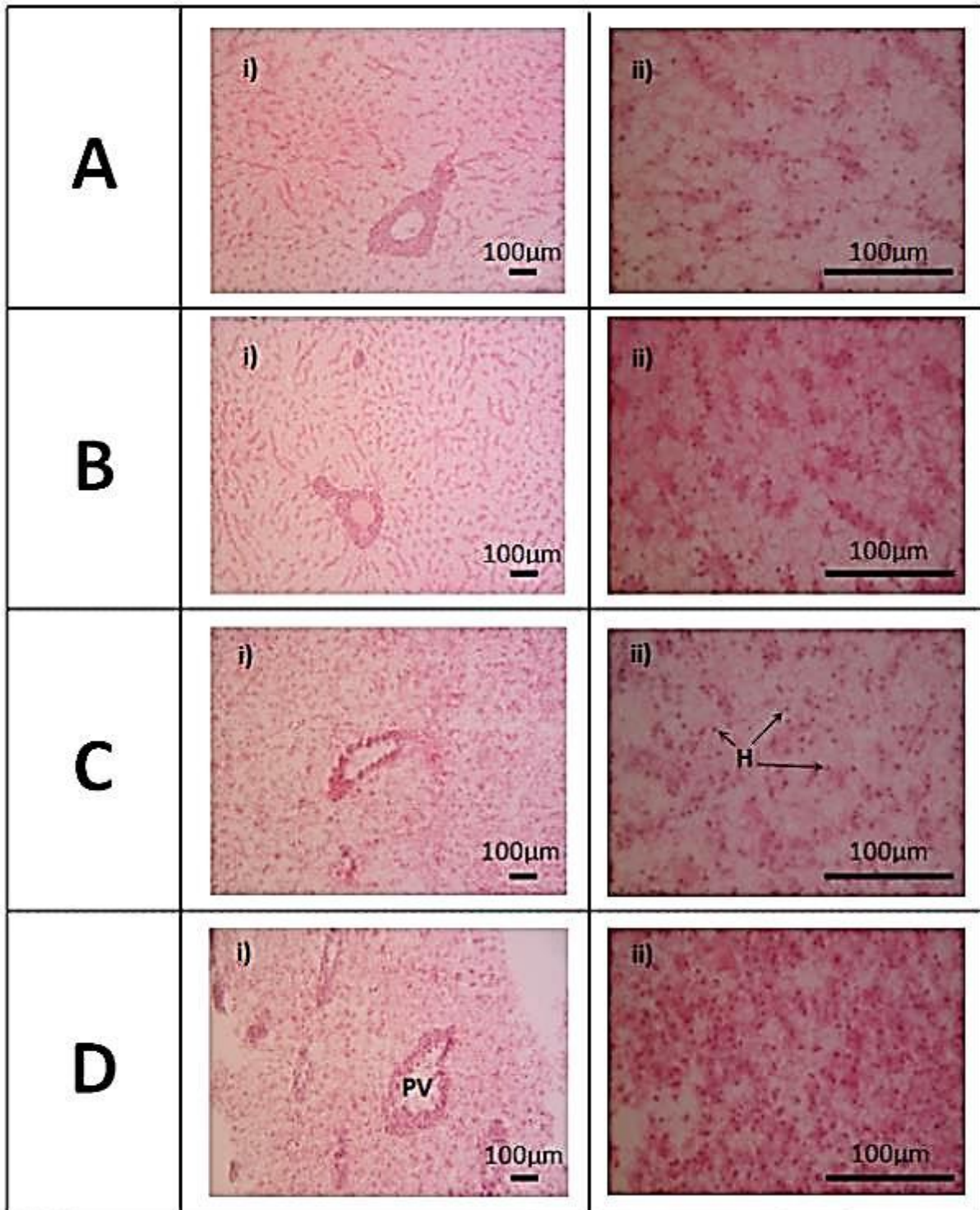
Figure 3.8 Example sections of *O. niloticus* liver micrographs, used for appraisal of hepatocyte nuclei counts from hepatic tissue samples.

Scale bars: i) = 100 μm and (ii) = 100 μm.

Treatment groups: (A) = Control diet, (B) = 15% inclusion, (C) = 30% inclusion, and (D) = 60% inclusion. Key: (H) hepatocytes, (PV) portal vein.
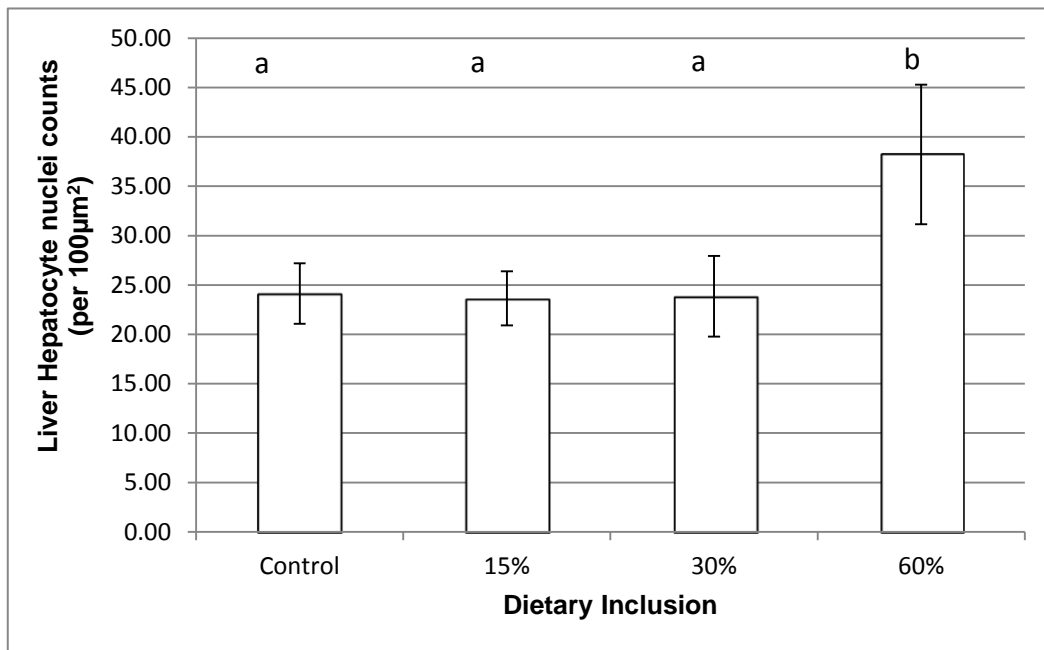
Figure 3.9 The effect of dietary *Chlorella* inclusion levels on liver hepatocyte nuclei counts from light microscope micrographs.

Means ± SD (*n*= 9) (*p* < 0.05)

Treatments that share the same superscript letter are not significantly different.

3.3.5 Photographic assessment of skin colouration

After the image capturing process, there was no apparent visual variance in skin colour between any of the groups (Figure 3.11). A preliminary red colouration assessment of caudal areas from each of the dietary groups (*n=12*) confirmed that no significant differences between mean red values were observed, and thus no further extensive analyses was undertaken (Figure 3.10).

Figure 3.10 Standardised photo appraisal of *O. niloticus* coloration differentiation after 35 days of feeding *Chlorella* inclusion diets.

Diet codes: (A) = Control diet, (B) = 15% inclusion, (C) = 30% inclusion, and (D) = 60% inclusion.
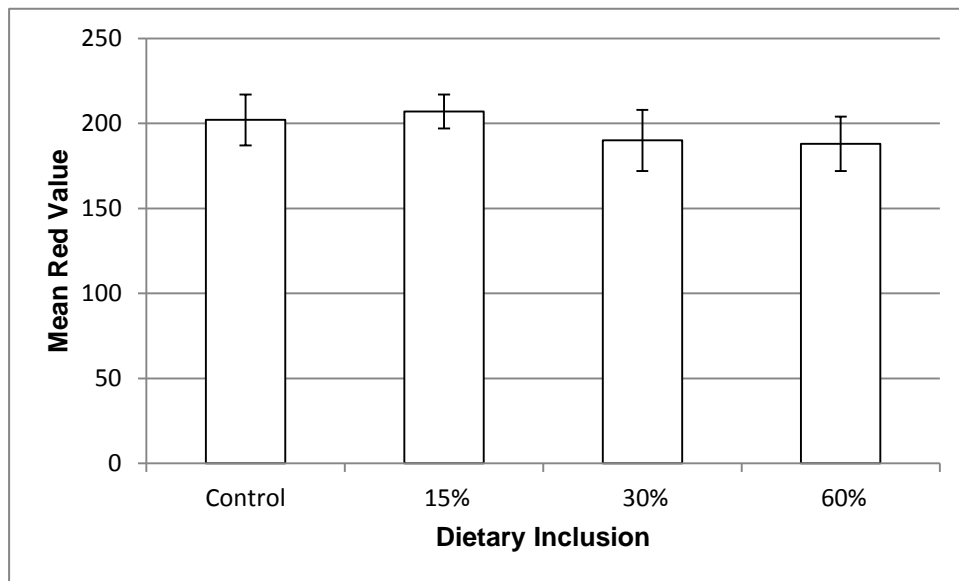
Figure 3.11 Results of photographic appraisal of red colour enhancement in *O. niloticus* in response to increasing dietary inclusion of *Chlorella* after 35 days.

Means ± SD (*n*= 9)

Error bars represent Standard Deviation. No significant differences observed ($p > 0.05$).

3.4 Discussion

Through the development of a thick protective cell wall, *Chlorella* spp. have become very resistant to changes in environmental conditions as well as achieving an astounding capacity for reproduction, resulting in cells of very high protein content (Loos and Meindl, 1982). Subsequently, this has led to the development of technologies for harnessing this protein source for human foods (Wikfors and Ohno, 2001). Initiated sixty years ago at the Massachusetts Institute of Technology (Little, 1953), the Carnegie team excelled in developing technology using enclosed culture systems or PBRs for the production of *Chlorella* species. Considerable progress has been achieved since then, predominantly by the Aquatic Species Program: 1980–1996 (Sheehan *et al.*, 1998), and now a large world-wide research and development effort is underway to extract and exploit protein and other high value cellular components from *Chlorella* spp (Benemann, 2013). The productivity in terms of biomass and lipid content of *Chlorella* can both be enhanced if specific culture conditions are applied (Al-lwayzy *et al.*, 2014); after harvesting, drying and lipid extraction processes have occurred, a residual by-product remains (algal meal). The ruptured cell retains components that may possibly support fish growth and health.

In the present study, the potential of three inclusion levels of a proprietary *Chlorella* sp. in semi-practical diets for *O. niloticus* was investigated. All diets were formulated to be isonitrogenous and iso-energetic, meeting requirements as put forward by Jauncey (1998a) in feeding tables for this fish species. It should be noted that the high inclusion diets of 30% and 60% resulted in a very hard pellet with a glossy exterior, unlike the control and 15% *Chlorella* pellets. All diets were readily accepted by the tilapia, although the 60% addition of *Chlorella* in this trial resulted in it taking longer to administer the feed.

The growth performance and feed utilisation efficiency in the current investigation was comparable with a study by Lupatsch (2013) where growth performance gradually deteriorated with increasing protein inclusion levels (30%, 60% and 100%) of *Chlorella* in diets for all-male *O. niloticus*, although this study replaced fishmeal directly within the dietary formulations. Appler and Jauncey (1983) noted similar findings to the current study, although in their investigation *Sarotherodon niloticus* fingerlings were utilised as the model species with incremental inclusions of *Cladophora glomerata* meal at 5%, 10% & 20% in replacement of fish meal protein. In this study, FCR increased significantly for the fish fed 60% *Chlorella* inclusion, indicating more feed was required to achieve weight gain. The condition factor (K) reflected this, serving as an indicator of physiological state of the fish in relation to its welfare in a research setting where variables are minimised. The control diet resulted in K of 2.22, in comparison to fish fed the highest inclusion level of *Chlorella* where K decreased to 1.67. This represents a decline in condition factor of the fish fed the highest algae supplement when compared to the control group, although all values were still in accordance and range reported by a number of studies utilising *O. niloticus* of above 1, for healthy tilapia (Tadesse *et al.*, 2003; El-Saidy and Saad, 2011; El-Serafy *et al.*, 2013).

The results from this study show that crude protein, lipid and ash content of the carcass were all significantly different to the control group at the 60% inclusion level of dried *Chlorella* biomass at the end of the 35 day study. The 15% inclusion level decreased carcass crude protein content slightly from the control , declining further in the 30% inclusion group, although not significantly different to the 15% group, with 60% inclusion revealing a significant difference to all others. Crude lipid content in the control group increased, but not significantly with the 15% inclusion level, declining thereafter for 30% and 60% inclusion

levels. Ash content increased with each algal inclusion level, significant at 60% inclusion, although reduced in all groups from the initial starting carcass composition of tilapia.

The decreases in carcass protein and lipid content may indicate less nutrients channelled into storage.

The experimental study provided evidence that 15% inclusion of the algae could be feasible. This seems to be the threshold, above which overall performance was impeded. It remains to be seen whether an optimum algal level below 15% could provide benefits to tilapia and be a cost-effective additive to the feed. The reduced performance of tilapia fed algal levels higher than 15% maybe due to a combination of reduced digestibility and inferior amino acid balance at higher substitution of other ingredients, namely soybean. Tartiel, et al. (2008) stated that *Chlorella* sp. was potentially deficient in sulphur containing amino acids (methionine) and high diet inclusion level maybe detrimental to growth in *O. niloticus*, as found in this study. Further, the cellulose from the thick extracellular polysaccharide wall of the *Chlorella* may interfere with intestinal assimilation of nutrients, possibly inducing an evacuative response prior to utilisation of feed for growth (Rezanka and Sigler, 2007). This was evident from the water of the closed recirculation system, which turned green after several days of feeding. Whether this was due to separation and elimination of algal cells within the buccal cavity and subsequent losses from the tilapia branchial basket or from direct faecal losses remain unclear. However, incomplete digestion is the likely cause.

Biological parameters such as HSI are sometimes indicative of health status, and the results from this study suggest that all of the values for HSI in microalgae fed groups are still well within the range of healthy, non-compromised fish (Min and Kang, 2008; El-Serafy *et al.*, 2013). The health related biomarkers investigated in this study, haemoglobin, haematocrit, RBC and WBC levels, were in-line with the normal ranges for healthy fish (Akinleye *et al.*,

2012), and were not significantly affected by dietary *Chlorella*. These findings, combined with acceptable survival levels, imply that the fish did not suffer from any toxicological effects in response to the ingestion of incorporated *Chlorella* cells at any level. This is in agreement with Cheunbarn and Cheunbarn, (2015) that studied the possibility of cultivating Chlorella for the use in dried feed for *O. niloticus* using vegetable and fruit canning industry waste treatment effluent, although inclusion levels were to a maximum of 3%, limited by cultivation techniques.

When conducting histological appraisals of morphological changes in *O. niloticus* intestinal tracts, most investigations are centred on the toxicological effects of heavy metals and/or environmental pollutants (Younis *et al.*, 2013; Abdel-Khalek, 2015; Guzman-Guillen *et al.*, 2015), or reporting on probiotic effects (Merrifield *et al.*, 2011). Those that are relevant, investigate other visceral organs such as the stomach, pancreas and spleen (Osman and Caceci, 1991), or else serve only as a base (Gargiulo *et al.*, 1998), similar to the control within a particular body of work, or are applying it to other model species of importance to aquaculture such as carp and rainbow trout (Dimitroglou *et al.*, 2008; Omar *et al.*, 2012), making comparisons of microalgae inclusions for *O. niloticus* challenging.

This current study of the mid-intestinal tract and liver at gross and ultrastructural levels revealed no impairment to health, with samples exhibiting good overall architecture and structure. A clear trend of increasing intestinal PR was observed in the fish fed with diets containing algae substitutions, indicating an increased internal absorptive surface area possibly brought about by more numerous mucosal folds, possibly exhibiting the adaptive potential of this organ.

In microalgae, carotenoids function as protection against oxidative damage and as accessory light-harvesting pigments, thus protecting the photosynthetic apparatus against photo-

damage (Sujak *et al.*, 1999). Fish cannot synthesise these carotenoids de novo, but instead require supplementation from dietary inputs. *Chlorella* spp. and *Spirulina* spp. are commonly incorporated into feeds for ornamental fish, where colouration and healthy appearance are the main value- adding attributes within the ornamental fish trade (Gouveia *et al.*, 2003; Rema *et al.*, 2003; Zatkova *et al.*, 2011).

However, no change in the red spectrum of skin colour was observed in tilapia fed Chlorella, when compared to the control group. Possible speculative reasons are that the carotenoids within the *Chlorella* cell may not have even been bioavailable, carotenoid molecules may have been metabolised before depositing them onto natural receptors in the skin or else the hybrid *Chlorella* sp. obtained from source, contained incorrect carotenoid groups suitable for skin colouration changes to occur. As carotenoids are lipid soluble pigments they may have been extracted from the algal meal at the time of lipid recovery or simply destroyed by processing of the algal meal or released into the RAS system from the 60% inclusion replicates due to rapid evacuation time though the fish in these tanks or else some feed components may have interfered with the release of carotenoids hindering the uptake by the intestinal mucosal cells as was reported by Shete and Quadro (2013), using a mammalian model in beta-carotene metabolism. Any future efforts should investigate the carotenoid composition of algal meal to aid improved understand of absorption and utilisation characteristics, alongside the assimilation of any microalgae carotenoid sources if they are to play a role in aquaculture and/or the ornamental trade.

3.5 Conclusions

The results from this study suggest that this proprietary *Chlorella* sp. evaluated will not function as a protein substitute at inclusion levels higher than 15% in moderate fish meal inclusion levels in tilapia feed. Further studies should concentrate on inclusion levels lower than 15%. The growth performance and feed utilisation indices gradually declined with increasing inclusions of microalgae. No signs of negative health or welfare issues were observed in any of the treatment groups throughout the 35 day feeding trial. Diets were readily accepted by all of the tilapia groups at the beginning of the trial, this gradually declined as the trial progressed with respect to the two higher inclusion diets. Even though reduced growth performance occurred with respect to the 60% inclusion level, all haematological parameters and histological assessments of gross and ultrastructural morphology appeared unaffected, as there were little to no significant differences discovered compared to the control reference diet. Further investigations are required to elucidate the use of defatted, proprietary *Chlorella* sp. strains in aquaculture diets for *O. niloticus*.

Chapter 4

General Discussion & Conclusions

4.1 Discussion

It is widely agreed that the global population is going to increase over the next few decades, adding to greater pressure on available protein resources and further depleting fresh water supplies (Pimentel and Pimentel, 2003; Luigi *et al.*, 2010). This, accompanied by the continued exhaustion of finite fossil fuel reserves and rising atmospheric CO_2 levels, has put microalgae biofuel production high on the agenda (Yuan and Xu, 2015). With this need for sustainable solutions in mind, a biotechnology industry sector has emerged over the last decade, focused on obtaining biofuel from natural organisms including unicellular algae, yeasts and bacteria (Adenle *et al.*, 2013). In consultation with a leading US algal producer, a unique opportunity arose to investigate the application of two selected hybrid microalgae products (*Nannochloropsis* sp. and *Chlorella* sp.) using tilapia, a major farmed fish species, as the experimental model.

Tilapias are a high value species with production expanding around the world to approximately 4.5 million metric tonnes per annum (Infofish, 2015). The increasing trend for the production of tilapia is attributable to their omnivorous nature, coupled with their robustness, speed of growth and tolerance of high stocking densities. Tilapia is also a good source of protein and versatile fish that easily fillet offering pure white flesh and good taste (Borresen, 2009). In line with the continuing quest for more cost-effective and sustainable feed for a variety of production systems, tilapias allow for rapid assessment of new feeding ingredients and dietary formulations and therefore are an excellent model for preliminary investigations prior to embarking on studies involving more expensive and exotic species (Floros *et al.*, 2010). These factors were the main rationale behind the current research, which sought to evaluate unicellular algal biomass by-products from the biofuel production

sector from a new perspective. This comes with a clear understanding that the production cost of algal biomass differs greatly, governed by the various production methodologies employed. Base costs for live algae (wet weight) are projected as $1.6 kg^{-1} ($1600 per mt) for open pond raceway production to $9.84 kg^{-1} ($98400 per mt) when closed PBR systems (Slade and Bauen, 2013) are applied. These costs are reducing per annum as biotechnology and infrastructure continue to progress and develop. As a consequence, any level of inclusion into linear least cost diet formulation software would be rejected as the economics are prohibitive at this current time, unless the biomass originates as a by-product from the algae, after oil extraction for the primary biofuel route.

The nutritional assessment of the algal test biomasses employed conformed to standard protocols and scientific principles associated with fish nutrition studies ; consistent with established fish nutrition feeding trial protocols, the scientific approach for the design and initiation of the two trials therefore conformed to good scientific practice, husbandry and welfare practices for fish (Hastein *et al.*, 2005). It should be noted that such practices and associated procedures were compliant with Home Office Guidance on the operation of the UK legislation (Home Office, 2013) for animals used in research under the Scientific Procedures Act (1986), as well as being approved by the ethical review body of Plymouth University. An effective dialogue was maintained with the producers of the dried microalgae biomass source in order to try and fully understand the relevant technical aspects and constraints of production for the algae in question. However, due to confidentiality agreements having been previously signed by all parties, all information relating to the exact blend of species used in production and the harvesting processes specifications of the products studied was unavailable.

A controlled experimental feeding trial, using high quality fry obtained from broodstock selected for 'YY' male genotypes through the application of basic genetic manipulation for production of nearly all male (>95%) progeny, was utilised and supplied by Fishgen (Fishgen, 2005). Experiments were conducted in small fibreglass tanks in a closed recirculation systems (RAS) with sufficient replication to allow scientific validation of the performance characteristics of fish fed experimental diets.

The literature suggests that the use of dry algal meals as a dietary component in compounded feeds has generally been limited to date, compared to the vast array of information available on the use of algae as part of live food systems which, due to their relatively low expense, are most common in fish and shell fish hatcheries. The notable exception, as reported in older literature, has been the use of *Spirulina* spp*.* (now classified *Arthrospira platensis* and *Arthrospira maxima*) in diets for a range of fish including carnivorous species such as rainbow trout (*Oncorhynchus mykiss*)(Guroy *et al.*, 2011) and red sea bream (*Pagrus major*)(Mustafa *et al.*, 1997; Nakagawa *et al.*, 2000), as well as omnivorous species such as common carp (*Cyprinus carpio*)(Nandeesha *et al.*, 1998) and applications for ornamental species such as red swordtails (*Xiphophorus helleri*)(James *et al.*, 2006).

The main aim of the first trial (described in Chapter two) was to evaluate dried, defatted microalgal biomass (*Nannochloropsis* sp.*) as a feed additive when incorporated into the diet at moderately low levels. This was achieved by the substitution of the soybean concentrate and corn starch in increments of 5% algae, formulated up to a maximum of 15% algal biomass incorporation, as these could be considered a realistic integration level for commonly practiced diet formulations used within industry (Ozorio *et al.*, 2012). This enabled the assessment of the practicalities of incorporating acceptably low levels of FM,

with a bias towards plant-based ingredients and dried microalgae and the effects of cold press extrusion within our pellet manufacturing process. Other factors were considered at this juncture, such as the general acceptance of pellets for size, particle size, texture and general palatability before commencement of the respective feed evaluation trials.

All growth performance and biological indices were found to be in accordance with other studies for tilapia when fed practical formulated diets (Abdel-Tawwab and Ahmad, 2009a; Bob-Manuel and Alfred-Ockiya, 2011; Koumi *et al.*, 2011). Survival was within acceptable limits at the end of the feeding trial period, although hierarchy confrontations were observed throughout the trial, with some dominant fish taking up governance within corners of the rectangular trial tanks. Such behaviour has been reported by Timmons *et al.*, (1998), and this could be avoided in any future trials by utilisation of circular culture tanks that enhance management strategies within production of food fish with reference to providing operation of a wide variety of rotational velocities to optimise fish health.

The quantitative histological appraisal of hepatocyte nuclei counts as an indicator of liver cell density showed an increase in all fish fed diets containing *Nannochloropsis*. Neither hypertrophy nor hyperplasia was observed in the livers of fish fed the algal diets. Other studies have revealed that alterations in number, size and shape of the hepatocyte nucleus can be due to contaminants or an increase of metabolic activity due to nutritional over-load (Figueiredo-Fernandes *et al.*, 2007), but there was no indication of nutritionally related pathology in this study, as inferred by the HSI data which was not significantly different between the groups.

Peripheral white blood cell (WBC) counts increased with *Nannochloropsis* inclusion. Differential cell counts revealed that granulocyte levels were primarily responsible for the elevated WBC population. The mechanisms responsible for stimulating the innate defence

are not fully understood, but a number of algae derived compounds and molecules have been reported to have immunostimulatory effects in fish (Hsu *et al.*, 2010; Cerezuela *et al.*, 2012; Zhang *et al.*, 2014). Their elevation in the algal fed fish in the current study may therefore be indicative of improved defence against pathogen challenge, which should be explored in future disease challenge studies. Similar immunostimulatory effects of the innate parameters are reported by several other authors who state that phagocytosis is stimulated by the oral administration of probiotics (Abdel-Tawwab and Ahmad, 2009b; Nagasawa *et al.*, 2014), that are a viable comparison with algae since they are single celled organisms (e.g. bacteria) that can be added to the diets of various cultured fish and shellfish.

By using both SEM and light microscopy, it was possible to gain an overall visualization of gross and ultra-structural morphology of the intestine, which is a dynamic and changeable organ. The only significant difference observed was an increase in intestinal mucosal folding in tilapia fed diets with an incorporated microalgae meal inclusion of 15%, when compared with the control group of tilapia. Teleosts such as tilapia are regarded as lower vertebrates, which possess simple longitudinal intestinal folds instead of the more complex intestinal villi found in higher vertebrate classes (Sastry, 1973). Consequently, it is the intestinal fold height that is regarded as the sign of the absorption capacity in these animals and positively correlates with nutrient uptake efficiency (Zhang *et al.*, 2013). Numerous studies have reported that probiotics have improved the intestinal wall thickness, intestinal mucosa height and microvilli density in aquatic animals (Pirarat *et al.*, 2011; Abid *et al.*, 2013; Sharifuzzaman *et al.*, 2014; Fang *et al.*, 2015), similar to findings of this study.

With respect to the general superficial appearance of tilapia, increased colouration of epithelial skin was the most notable finding of the first trial, with increases in red colouration visually observed after only few weeks of feeding diets with algae inclusion. At

the end of the trial, data collected suggests that the carotenoids (although, un-identified for this microalgae sample) supplied by the *Nannochloropsis* cells had a direct positive influence on the red colouration of the skin, based on visual comparative analysis with control fish. These observations could potentially have a positive impact for cultured tilapia species sales in terms of human consumption, adding value and greater consumer acceptability and appeal due to observable red pigmentation enhancement (Alfnes *et al.*, 2006). As colour is one of the main contributing factors in determining the value of fish for the food industry, it will become increasingly necessary to develop a process to evaluate the effects of different sources and levels of pigments and dietary supplements on the colour intensity of muscle and fish skin (Fries *et al.*, 2015).   Furthermore, augmentation of red colouration within the ornamental fish industry (inclusive of the aquarium trade) possesses considerable potential, as this market is expanding at approximately 14% per annum and in 2012 was estimated to be worth four billion dollars globally (Lango Reynoso *et al.*, 2012). Given that fish are unable to synthesise carotenoids *de novo*, only a few studies have evaluated the effects of dietary pigments on the skin colour of ornamental fish (Sun *et al.*, 2012; Kouba *et al.*, 2013). With high-value species in the ornamental trade such as Koi carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), the emphasis should be on achieving high levels of skin pigmentation which, together with body shape, fin shape and body size, are the most important quality criteria informing their market value (Gouveia *et al.*, 2003).

Researchers and farmers alike require a simple rapid and accurate method of colour analysis; the photographic approach described herein provides an alternative to expensive, high technology solutions such as reflectance spectrophotometric measurements, High Performance Liquid Chromatography (HPLC) or complex carotenoid extraction assays (Chatzifotis *et al.*, 2005) that are not necessarily widely available. Whilst this may represent

a more cost effective way of detecting coloration changes, refinement of this methodology is required if wider use is to be considered.

The second trial (Chapter three) investigated an alternative proprietary dried, defatted, commercially manufactured *Chlorella* sp.; *Nannochloropsis* was requested as a continuation from the first study, but this was not possible due to availability issues and production limitations. This trial utilised higher inclusion levels in order to identify potential adverse effects such as nutritional deficiency or toxicological effects. Therefore, there was a requirement to examine visceral organs and tissue in conjunction with growth and health parameters. By applying standard formulation practices microalga cake extraction derived from biofuel production was used to achieve balanced protein and calorific diet formulations for appraisal in tilapia. A slightly lower percentage of FM inclusion was used for this second trial, necessitating the inclusion of a lower level fish oil (FO) to be applied to achieve iso-energetic formulations. Soybean meal was reduced and subsequently substituted with pea protein (Lysamine®) concentrate (PPC) with each inclusion level of *Chlorella* sp. at 15%, 30% and 60%, with the intention of appraising a more practical diet formulation which with theoretically equal plant protein sources (Zhang *et al.*, 2012). PPC was introduced into feeds due to reported negatives arising from the use of soybean in aquaculture diets (Gatlin *et al.*, 2007). For example, soybean are staple food crop for humans, their common use for food-producing animals directly competes with their allocation for human consumption (Lum *et al.*, 2013), there is a reported high carbon footprint of soybean meal from importation from the USA and Brazil, the largest producers (Lathuilliere *et al.*, 2014). Additionally there is also a reported reduction in importation from the USA due to the resistance to genetically modified (GM) soybean in European markets (Mezzelani *et al.*, 2002) and the fact that soybean meal trade is forecast to rise necessitating

an increase in cost, driven by demand in Southeast Asia (Muhammad, 2015). PPC also contains fewer ANFs than soybean (Valencia *et al.*, 2008), together with the PPC containing a lysine content equal to fish meal; lysine is considered to be one of the first limiting essential amino acids when replacing FM with plant proteins (Tomoskozi *et al.*, 2001).

Within this study significant differences were observed with regards to all growth performance and biological indices parameters at the conclusion of the trial in conjunction with an overall >95% survival. The trial was terminated after 35 days due to the control group reaching a successful tripling of body weight together with a disproportionate retardation of growth in the 60% inclusion level (it was speculated that this may have begun to compromise the physiological functions of the fish).

It is conceivable that the impedance of growth and subsequently the biological indices investigated were due to high fibre or indigestible matter (such as cellulose) content of the algae, known to be present in the cell wall of *Chlorella* sp. (Rodrigues and da Silva Bon, 2011). Poston (1986) investigated the response of lake trout (*Salvelinus namaycush*) and rainbow trout to dietary cellulose, reporting that fish growth decreased linearly as quantities of cellulose increased. The investigation carried out by Poston (1986) and a separate study by Bromley and Adkins (1984) described similar carcass composition observations to those described in this study, in that whole carcasses of fish fed higher cellulose levels contained significantly less lipid and protein. Both investigations also reported the release of excessive quantities of excreta caused by indigestible wastes that subsequently increased pollution of the system water supply. Although fibre analysis was attempted with the supplied *Chlorella* sample, no result was achievable due to the fine powder form of the microalgae, which after "in house" processing was too fine for analysis using the fibre tech system that requires retention of particulate matter. An alternative

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theory for the retarded growth and subsequent negative effects on the biological indices could be that these effects are due to the retention of available nutrients within the algal cell wall owing to low digestibility due to insufficient structural damage occurring during the harvesting process of some cells, as SEM micrographs appear to show possible whole cells (as observed in Figure 3.1).

All haematological parameters investigated were verified to be within acceptable limits for this species as described by Hrubec *et al.* (2000), implying that the inclusion of algae up to 15% for tilapia did not adversely alter their main blood parameters.

Fish liver functions include the assimilation of nutrients and maintenance of the animals metabolic homeostasis; this includes the processing of carbohydrates, proteins, lipids and vitamins, although many authors frequently report alterations to the liver with regard to the intake of substances of toxicological significance (Figueiredo-Fernandes *et al.*, 2007; Caruso *et al.*, 2013; Latif *et al.*, 2014; Puerto *et al.*, 2014). However, only a few researchers have reported histological changes of the liver in response to novel feed ingredients (Dimitroglou *et al.*, 2010; Guroy *et al.*, 2011; Omar *et al.*, 2012). Analysis of histological samples of liver at the gross microscopic level in this study showed no differences between groups when determining hepatocyte nuclei counts, in conjunction with broader observations; all specimens prepared had well-defined architecture, with no observable paucities in cellular function, suggesting that the physiological function of this organ was unaffected in all groups.

Determination of peripheral skin colour showed no differences between control fish and any of the fish fed with *Chlorella* inclusion diets. This result was unexpected, as previous use of this new analytical method described in Chapter two showed a positive change in the integument coloration when *Nannochloropsis* was applied at lower inclusion levels. It is

understood that colouration of the skin in poikilothermic vertebrates is influenced by six kinds of pigmentation cells, inclusively termed chromatophores (Fujii, 2000). It is possible that the method of photographic assessment applied in these studies were too simplistic for such complex chromatic system alterations to be observed. Additionally, the main carotenoids produced by *Chlorella* spp. are canthaxanthin and astaxanthin; these are already widely used as pigments in feeds to colour salmonid fish flesh (Guedes et al., 2011) and will not likely affect the dendritic chromatophores of *O. niloticus* (Leclercq *et al.*, 2010). Further studies and refinement would be necessary to confirm this, accompanied by assessment of specific carotenoid content and bio-accessibility of microalga samples studied (O'Sullivan *et al.*, 2011). The present research only investigated the surface integument colour; future work could also extend to evaluate flesh colouration, taste and texture testing (sensory panel testing), especially when marine algal species are considered, as this may enhance the taste of perceived bland freshwater species due to the presence of bromophenolic compounds (Kawai, 1996). Although it should be noted that, according to European Union, Regulation (EC) No 1831/2003 (2003), only additives that have been through an authorisation procedure may be placed on the market. Authorisations are granted for specific animal species, specific conditions of use and for ten year periods. The European Food Safety Authority (EFSA) is responsible for conducting the evaluation of the data submitted requesting authorisations. After a favourable review from the EFSA, the Commission prepares a draft regulation to grant authorisation, following the procedure involving Member States within the Standing Committee on Plants, Animals, Food and Feed (Gilsenan, 2011). This process must be fulfilled prior to any algae going to market or even before being evaluated by expensive taste testing panels. This would also ensure any

negative consequence of feeding microalgae to aquaculture species of interest was called to attention before reaching market.

Further digestibility studies would be extremely beneficial to evaluate protein digestibility of microalgae components to gain measurements of digestible protein, amino acids and energy for precise formulations based on digestible nutrients in the algal biomass. As reported by Ribeiro *et al.*, (2012) the determination of digestibility coefficients is usually based on faecal measurements. However, as settlement collection is the most commonly used faecal collection technique, faeces fragmentation and leaching of faecal components often results in an overestimation of digestibility values. Alternative techniques that avoid this require removing the fish from the water and collecting faecal samples directly from the distal intestinal region, but this approach is not possible with this species, as tilapia possess a very long gastro-intestinal tract (Fontainhas-Fernandes *et al.*, 1999; Ribeiro *et al.*, 2012; Albers Koch *et al.*, 2014; Koch *et al.*, 2016). As a consequence, extraction of faecal matter for analysis would necessitate euthanasia of the studied animal and this would not support the implementation of the 3Rs (Replacement, Reduction & Refinement) as part of conditional practices within our animal research strategy.

Any future work should incorporate all of the parameters explored in the two studies presented herein as this would assist in providing a comprehensive evaluation of dietary inclusion of microalgae cells. In addition, profiling of amino acids, long chain fatty acids and fibre content would enhance understanding of dietary formulation for nutritionally complex fish species. Future studies should seek to identify the most beneficial components of the microalgae cells in order to provide targeted nutrition; there may be merit in utilizing two or more strains / species concurrently in order to achieve more balanced formulated diets, this

in consort with an analysis of gut microbial activity as an assessment of microbiological indices from a probiotic/prebiotic perspective.

The assessment of other species of interest to the aquaculture industry, as well as larger scale studies of tilapia would be beneficial in providing a more comprehensive understanding of the effects of dietary inclusion of microalgae, and would build on the current research reported herein.

4.2 Conclusions

The findings of these collective studies have demonstrated that tilapia are a rapid growing fish with an ability to respond favourably to low dietary inclusion of hybrid *Nannochloropsis* sp. and *Chlorella* sp. in formulated balanced diets. However there are restrictions to substituting plant proteins with these algal by-products at higher levels.

The findings showed that the diets provided minimal effects on any of the biological indices for tilapia. Low level inclusion of both algae hybrid samples showed a potential positive effect on gut morphology (possibly by increased absorptive surface area) and a positive influence on haematological indices by way of increased granulocyte activity, indicating an enhancement of phagocyte function as part of the innate immune response system. It should be noted that this study focused on juvenile fish for growth feed performance assessments. Future investigations must be directed to the grower and finishing stage where the microalgal incorporation will be possibly more cost effective and add value at harvest.

The innovative low cost photographic approach to appraising colouration changes of integument cells of *O. niloticus* showed promising results in the first trial, revealing a clear increase in red colouration of the caudal area of red tilapia. Even though no such changes in

skin colouration were observed in the second trial, future studies at low level inclusion of *Chorella* sp. should be continued so as to identify any previously unseen beneficial effects of elevated carotenoid levels on pigmentation and general health status.

The project has demonstrated that a fast growing species such as tilapia can be an effective model to evaluate a novel algal feed ingredient within defined inclusion levels. The algal biomass tested in the respective feeding trials effectively substituted for the plant ingredients (soybean) without any detrimental effect on health and status.

It is hoped that these preliminary studies may provide a useful platform for increasing the incorporation of by-product microalgae cake from a growing biofuel industry into the diets of a range of popular aquaculture species.

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Appendix

SOP -Determination of Lysozyme activity using a turbidimetric assay

Lysozyme is present in the serum and mucus of fish but is particularly associated with leukocytes and leukocyte-rich tissues e.g. kidney, spleen and gut. Lysozyme has anti-bacterial activities, particularly on gram positive bacteria by causing lysis and may also act as an opsonin. Lysosyme is capable of lysing important fish pathogens: *Vibrio anguillarium, Yersinia ruckeri* and *Aeromonas hydrophila.* The assay is based upon the lysis of the Gram positive bacterium *Micrococcus lysodeikticus* according to Ellis, (1990), which is obtained freeze dried.

EQUIPMENT
- Multichannel pipette
- Micropipettes and tips
- Microplate reader
- Microplate (flat bottomed) (3ml)
- Incubator

CHEMICALS AND REAGENTS
Fish serum
Micrococcus lysodeikticus	SIGMA M3770
$Na_2HPO_4.2H_2O$	BDH 4575K
$NaH_2PO_4.2H_2O$	BDH 310324

BUFFERS
Sodium phosphate buffer (SPB) (0.04 M, pH 5.8 trout; pH 6.3 Atlantic salmon)

Make a 0.2 M solution of $NaH_2PO_4.2H_2O$ by dissolving 31.20g in 1L dist. H_2O – **STOCK A** .

Make a 0.2 M solution of $Na_2HPO_4.2H_2O$ by dissolving 35.59g in 1L dist. H_2O – **STOCK B**

Mix the following amounts of each solution to achieve the desired pH:

STOCK A (ml)	STOCK B (ml)	pH
92.0	8.0	5.8
81.5	18.5	6.2
28.0	72	7.2

This will give you 100 ml of a 0.2 M solution. Dilute with 100 ml of dist. H_2O to give a 0.1M

SPB solution (final volume 200 ml) N.B. To achieve a 0.04 M SPB for the lysozyme, make up

your desired volume of the 0.1 M pH 5.8 solution and make a 1:2.5 dilution.

PROCEDURE

- Serum may be used fresh or after storage at -20°C

- Make up 50 ml (enough for forty samples) sodium phosphate buffer (0.04 M, pH 5.8/6.2) and place in incubator for 30 min at 25°C

- Prepare a suspension of *M. lysodeikticus* (0.2 mg ml–1) in sodium phosphate buffer

- Add 200 µl of sodium phosphate buffer (without bacteria) to columns 11 and 12 of microplate (– background control)

- Add sample (10 µl) to each of four wells (set up plate as in Figure 1)

- Add 190 µl of sodium phosphate buffer to all wells (except columns 11 and 12)

- Measure the reduction in absorbance at 540 nm at 1 min and 5 min. After first reading place plate in incubator 25°C

A unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance at 0.001/min.

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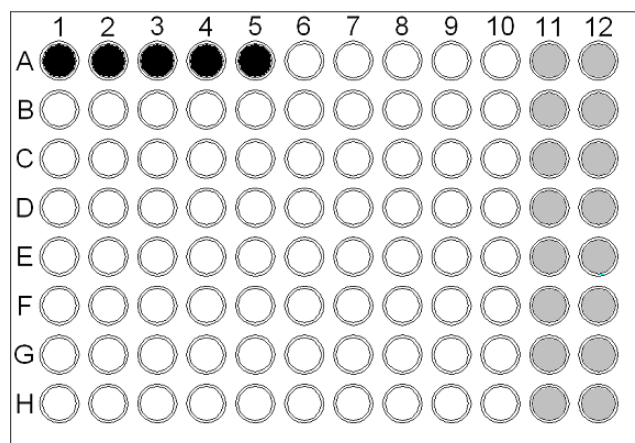
Figure 1. Plate set up for lysozyme assay. Samples are carried out in quintuplet with 10µl of sample/well. Sodium phosphate buffer must have been warmed to 25°C before bacteria are added.