Yellowtail Kingfish Growth Response, Flesh Quality and PUFA Content through Microalgae Formulated Aqua-Feeds

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Contents

I. Abstract

Yellowtail Kingfish is an emerging aquaculture species, and there is an increase in the demand for the fish, both for recreational fishing and for export purposes. This has led to the growth of the aquaculture industry that specialises in culture of yellowtail kingfish in South Australia. Currently very little is known about the physiological properties of yellowtail kingfish, and most data that has been generated on the fish has primarily been in Japan, where most of the scientific articles are written in Japanese. There is currently very limited supply of microalgae in Australia for use in commercial applications, and development is underway to encourage growth of the microalgae industry with a specific focus on developing a biorefinery system. One of the main aspects of the biorefinery system will be to use waste generated by the production of biofuels in other industries which will provide value addition to the waste products from the production of biodiesel.

One of the expected waste products of lipid extraction from the production of biofuels will be microalgae proteins, and the idea is to use the microalgae derived proteins for the growing yellowtail kingfish industry. The aims of this study were to i) use cultured/commercially available microalgae biomass as a an alternative protein source for yellowtail kingfish; ii) to examine the palatability of microalgae in fish feed; and iii) to observe any changes in the growth, performance and fillet quality of the yellowtail kingfish. The expected benefits are that the algae will provide a significant supply of antioxidants that will improve the flesh quality of the yellowtail kingfish.

Three diet formulations were devised for this study, and these diet formulations were the control diet made by Ridleys™, one of the largest aquafeed suppliers in Australia, and the other two diets were based on the Ridleys™ diets with fish meal being replaced by

spirulina in two levels resulting in diets that contain 20% *Spirulina* and 30% *Spirulina*. Feed trials were conducted for eight weeks, followed by biomass growth analysis and biochemical properties of the fillets obtained from the fish that were provided the dietary formulations.

The outcome of the feed trials was that there were no significant differences between growth rates of the fish fed the three feed formulations. The effects of the diets on the biochemical properties of the yellowtail kingfish fillets include:

a. Change in fillet colour, as fish fed the control diet had a slightly redder appearance but not significantly different from the microalgae substitution diet

b. Reduced lipid oxidation rates in refrigerated fillets, with fed the control diet showing the same oxidation levels as 20% *Spirulina* diet and 30% *Spirulina* diet with the highest oxidation levels, mainly attributed to a relatively lower feed intake level.

The results of this study indicate that the performance of the microalgae formulated feed is comparable to the control diet. There were significant differences between feed intake, but their effects of on the growth and fillet quality were not significantly different. Secondly, mortality rates were lower in fish fed microalgae substituted diets as compared to fish fed the control diet. Thirdly, observed antioxidant effects were not significantly different between the three dietary treatments, despite the significantly different feed intake levels.

These are indicators that the microalgae formulated feeds have a comparable performance to the control diet, this may suggest that if development of microalgae substituted diets progresses they may have the same performance if not better, than the

control diet. Results obtained in this study may warrant further investigation into developing a more palatable taste to allow for increased palatability and also investigate further on how carotenoids present in the microalgae may affect flesh quality of the fish.

This study has provided an opportunity to further explore the use of microalgae in fish feed particularly yellowtail kingfish and this has the potential provide low cost protein source alternatives for use in aquafeeds.

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1 Introduction

The marine fish *Seriola lalandi* (Yellowtail Kingfish) is found in almost all parts of the globe particularly Southern Australia , South Africa, Japan, New Zealand, and the USA in the regions with similar climatic conditions, and it is considered to be a circumglobal species (Poortenaar, 2001) It belongs to a Carangidae family of fish and it is commonly found inhabiting cool, temperate to warm water close to the coast line (Aquaculture SA, 2002). Several observations in New Zealand have identified YTK to be a potentially valuable organism for use in aquaculture (Poortenaar et al., 2001, Sakakura, 2004). Due to the increasing demand of the fish, it is seen as an organism which has a high potential for aquaculture, and early stages of culture and technologies are currently being developed in South Australia.

Due to the increasing demand for YTK, efforts are being made to meet the demand posed by the increasing consumption through intensive through intensive production so as to reduce the pressure on wild fisheries (NSW Wild Fisheries and Research Program, 2007). The increase in the global demand for fish oil and fish meal has resulted in their price increase and considerable effort has been concentrated on developing fish meal free diets to provide a cost reduction on the production of aquafeeds. There is also a need to develop renewable protein sources for use aquafeeds, but this has thus far yielded very little knowledge on the physiological impact and use of plant protein sources for aquafeeds (Chebakki K, 2010). Several potential avenues of exploration have been the use of microalgae as protein and lipid sources in aquafeeds, but they have largely been limited by the cost of producing microalgae biomass which is currently very high, and this means that adding any products derived from microalgae will result in very expensive feeds (Mata et al., 2010)Although microalgae can be costly to culture, the development of a biorefinery could provide a more economically feasible solution as more valuable products are derived

from the dry microalgae biomass, which could provide economies of scale that provide cheaper alternative protein sources for use in aquafeeds.

1.1 Feeding microalgae to fish

Various marine and fresh water fish species consume microalgae to derive particular nutrients such as proteins and long-chain Polyunsaturated Fatty Acids (lcPUFAs) (Stanley, 1976). Aquaculture practices often incorporate microalgae cultures in the feed of some species of fish in culture. The most commonly used microalgae species in the aquaculture industry are *Isochrysis*, Chlorrella, *Spirulina*, and Nannochloropsis spp.(de la Noue, 1988).

Various studies on the effect of inclusion of microalgae in aquafeeds have been carried out. There are limitations on the data that is currently available, and a relatively small proportion of it has been carried out on feeding freshwater microalgae to marine fish and vice versa. Microalgae are known to have a high nutrient content and their use in animal feeds makes them more attractive as a nutrient source, but the abundance of a specific nutrient is only specific to each particular microalgae species(Harun et al., 2010).

Investigations on the use of microalgae as nutrient sources for fish have standardised the nutritional content of the control feed with the variations in substitution levels of the microalgae in order to investigate the effect of the use of microalgae as a nutrient source in fish meal(Glencross, 2009).This current study will follow the same methodology when investigating the use of microalgal biomass in fish feed. Several studies conducted to investigate responses of fish fed microalgae have shown mixed results amongst different fish species. Studies by Stanley & Jones (1975) had investigated the food conversion and growth of *Ictiobus cyprinellus* (bigmouth buffalo), *Tilapia aurea* (blue tilapia) and *Ctenopharyngodon idella* (grass carp) fed *Spirulina platensis*. The studies have shown that the weight change in the fish was directly proportional to the feeding rate, which eliminates

feed selectivity from the use of microalgae as an insignificant contributing factor to the growth rates of exhibited by the fish.

Based on this information, it can be hypothesized that feed selectivity will not be a significant contributing factor towards the feeding rates of the fish fed the different microalgae formulated feeds. Several studies that investigate the use of microalgae in fish feed have been conducted to support this hypothesis.

Studies by El Sayed (1994) showed that *Rabdosargus sarba* (silver seabream fry) fed *spirulina* meal (containing *Spirulina maxima*) exhibited growth rates comparable to growth rates of the fish when fed fish meal. The study suggests that *spirulina* meal may be a great substitute for fish meal up to an inclusion level of 75%. However at 100% inclusion levels the fish did not accept the diet well and as such were excluded from the study. The growth rate responses of the fish were found to be species specific, and the current study will provide a number of opportunities to obtain novel data on the use of microalgae, particularly *Spirulina* sp., on growth characteristics of a marine fish species that has different behavioral patterns, psychological characteristics and feed preferences from fresh water fish species.

Nandeesha *et al.,* (1998) have fed diets to Cyprinus carpio L. that contain inclusions of *Spirulina platensis* of different levels. The results indicated that the feed conversion ratio (FCR) and the specific growth rate (SCR) were not significantly affected, and there was an improvement in the net protein retention (NPR). This was attributed to the high protein content and essential amino acids of the microalgae. The proteins in the diet had the same digestibility as the control diet and they observed the maximum digestibility at *S.plantensis* inclusion levels of 50%, which resulted in a protein content of 28% in the diet. Based on these findings, the different formulations that will be generated for the feed trials in this study will have minimal effect on the growth properties of the fish provided the feeding

rates will be consistent with the maintenance levels that are currently used for culturing the fish.

1.2 Microalgae Culture and Potential

There is a wide and varied number of species of microalgae, which are autotrophic microorganisms able to produce a wide variety of primary and secondary metabolites which may have significant value to the biotechnology industry (Harun et al., 2010, Molina Grima et al., 2003). Microalgae are found in a wide variety of habitats and some are found to be living symbiotically with other organisms. The photosynthetic nature of the microalgae make it a versatile and robust organism, which is able to produce large amounts of biomass and secondary metabolites from relatively simple precursor compounds, these include amino acids, proteins, antioxidants, carbohydrates, and lipids (de la Noue & Pauw, 1988; Harwood & Guschina 2009; Brennan & Owende, 2010).

The photosynthetic nature of the microalgae means that the culture of the microorganism does not require large amounts of a source to be included in the culture media. This gives large scale culture of microalgae great potential for commercial application. There is a vast array for the use of large scale cultures of microalgae for commercial applications (Harun et al., 2010, de la Noue, 1988, Molina Grima et al., 2003).

1.3 Cultivation Systems for large scale biomass production

There are two general systems that are used for the large scale cultivation of microalgae; these are the open pond systems, and the closed photobioreactor systems. Within these two systems there are varied types of designs, each with their own inherent characteristics but both with the goal of replicating the ideal growth conditions of the organism(Brennan and Owende, 2010, Harun et al., 2010).

Open pond systems are made up of a closed loop channel in which the culture media circulates through and are generally set up outdoors, where they are open to the elements to utilise the atmospheric light and carbon dioxide. The major design types of open culture systems are shallow ponds which normally cover a large surface area; tanks; circular ponds; and raceway ponds (Borowitzka, 1999). Their simplicity lowers their cost of operation, but this comes at a sacrifice of control over the culture conditions (Brennan & Owende, 2010; Harun et al., 2010; Mata et al., 2010). The productivity rates usually depend on the characteristics of the culture system. To achieve this, they will usually operate at depths of between 20 – 50cm depending on the culture strain and the light regime. A paddle that creates a flow velocity of about 30cm/s also increases the maximum biomass yield by creating a homogenised cell mixture which ensures even nutrient distribution (Borowitzka, 1999; van Beilen, 2009). It is of importance that a culture system of this type is not prone to contamination, often this is carried out by growing a strain of microalgae that has high growth rates and is very robust. Prevention of contamination is also usually aided by use of highly saline media through the addition of NaCl to about 15 -25% (Borowitzka, 1999) or sea water as the media base to create a highly selective environment of the microalgae being cultured (Mata et al., 2010). There are a large number of scientific articles currently published that claim productivities of outdoor cultures to be of proportions that are not realistically achievable with the current set ups of outdoor cultures. There are some limitations to the outdoor open air culture systems, which are likely to impede these high productivities mainly temperature drops during the night time, and a decreased photoperiod during changes in the diurnal period of different seasons. The effect of low light conditions results in a diminished growth rate for the microalgae growing in culture(Sandnes, 2005) There is also another condition called photo-inhibition (Bannister, 1979; Aiba, 1982) as cited in Grima et al, (1999) in which a specific irradiance level inhibits the growth rates of the

microalgae. This is a common occurrence in outdoor culture systems in which the microalgae growth is inhibited by extreme sunlight conditions, particularly during midday.

Closed photobioreactor systems on the other hand have greater complexity and offer greater control of the culture parameters over the conventional outdoor open air culture systems (Brennan & Owende, 2010). There are generally two types of photobioreactors namely: tubular and flat panels, each with the same goal of attaining a monoseptic culture that is free from any contaminants that may compromise the culture (Grima et al.,1999). Another important aspect of the design of the photobioreactor is the geometry, which can either be in a vertical, horizontal, or inclined plane configuration; they all allow for the minimisation of energy use during the culture period, in which the greater mass transfer of the vertical configuration allows for the lower energy use (Harun et al., 2010).

These systems are very ideal for culture of more 'sensitive' organisms that are susceptible to contamination by other algae, protozoan or atmospheric elements (Borowitzka, 1999). Photobioreactors are usually run indoors under artificial light, and therefore this means that there is greater control over the light regime, temperature, CO_2 levels, nutrient levels and dilution rates for continuous culture systems. The use of mathematical modeling techniques for the design of photobioreactors means that they can be designed according to the use for which it is intended, this means that photobioreactors can be used designed in such a way that accurately models the desired growth parameters of the microalgae strain of interest by addressing issues as suggested by Wiesmann et al., (1988) such as: (i) efficient provision of high quality light (ii) & (iii) efficient and non interrupted gaseous exchange system, where carbon dioxide is supplied and oxygen that many be toxic to the culture is removed (iv) and a sensible scalability of the photobioreactor technology. Addressing these issues is important in considering the design of the photobioreactor (Grima et al.,1999). Greater control of

these parameters through the use of photobioreactors in microalgae culture means that they have greater growth rates and generally achieve relatively high cell densities within the culture media (Brennan & Owende, 2010; Borowitzka, 1999; Grima et al.,1999).

There are drawbacks to the use of photobioreactors in large scale culture of microalgae, the main drawback being the scalability of the reactor (Grima et al.,1999). Scaling up each photobioreactor means scaling up almost all of its components, and there is a cost associated with each of the components which raises the cost of operation of each of the photobioreactors. The cost implication of scaling up does not yield a cheaper method of biomass production either, because new problems arise which limit the maximum cell concentration that can be attained within each photobioreactor (van Beilen, 2010). The first problem that arises from increasing the biomass concentration in a large scale reactor is the ability to control the culture parameters. An increased cell density means the illumination of the culture broth is not constant due to the effects of shading by other cells closer to the illumination source which results in less light being captured by each cell (Sandes et al, 2005). An increased cell density will also result in fouling of the components that are within the photobioreactor, such as the impeller and the nozzles that supply the CO_2 , nutrients, and the outlet for harvesting the culture broth. There is also the problem of O_2 accumulation in these closed systems, and this can lead to a decline in growth rates and photosynthetic efficiency of the organisms in the culture broth due to reaction oxygen species (Ganesh et al., 2006).

1.4 Harvesting microalgae

In the production process of algal biomass the cost of recovery of the biomass will be attributed to about 20 – 30% of the total cost of production (Pushparaj et al., 1993). It is

therefore imperative to develop and utilise an efficient and yet cost effective method of biomass recovery. There are several methods that can be employed to harvest microalgae biomass and these include, flocculation (Sukenik et al., 1988; Knuckley et al., 2006; Divakaran & Pilai, 2002); centrifugation (Grima et al., 2003) and membrane separation techniques (Rossignol et al., 1999; Zhang et al., 2010) all of which can be used in different combinations at the different stages of the harvesting process to improve harvesting efficiency (Grima et al., 2003). In this literature review we shall examine each process in a little bit more detail and we shall treat each process as a separate entity and how it can tie into the bigger picture of the recovery of microalgae biomass in a typical large scale setup.

1.4.1 Flocculation

The initial step in the separation of the microalgae from the culture media is flocculation (Bilanovic, 1988). This step allows for the significant removal of the culture media which reduces the volume and effort required to further process the microalgae biomass (Harun et al., 2010). There are several methods of inducing flocculation of the microalgal cells, and these methods primarily utilise the surface charge of the microalgae to draw each of the microalgae cells closer together to form "clumps" which makes them easier to deal with (Grima et al., 2003). One of the methods involves the addition of compounds known as flocculants which can be multivalent metal salts such as ferric chloride ($FeCl_3$), aluminium sulphate (Al_2SO_3) or ferric sulphate $Fe_2(SO_4)_3$; cationic polymers or polyelectrolytes which are either natural such as chitosan, which is usually obtained by deacetylation of chitin that is obtained from the exoskeleton of crustaceans or synthetic such as Zetag 63 and Zetag 92 (Harun et al., 2010; Bilanovic et al., 1988; Morales et al.,1985).

The addition of flocculants does not result in the same flocculation efficiency for any microalgal strain grown in any media. There are other parameters that influence the

flocculation of the microalgae under addition of flocculants (Sukenik, 1988). Studies have shown that in brackish and sea water based media, the flocculation efficiency was below 30% by using multivalent metal salts and these problems were attributed to the high salinity and the pH of the media (Knuckey et al., 2006). Several optimisation strategies were employed to improve the flocculation efficiency, and it was found that increasing the pH of the media and using natural polymers such as chitosan resulted in an increased flocculation efficiency, this has the potential to lower the costs of flocculation as less flocculant can be used to achieve the same amount of flocculation (Morales et al., 1985).

There are disadvantages to using flocculants to concentrate microalgae biomass, and which results in a potentially lower quality of biomass depending on its intended use (Knuckey et al., 2006). An example would be biomass that is intended for use as an inclusion in a meal diet of a particular organism. The use of naturally derived compounds such as chitosan can be expensive due to the high cost nature of obtaining it and its low flocculating power in high salinity and low pH media (Grima et al., 2003). Poleman et al. (1997) report a potentially useful method of flocculation that does not require the use of flocculants. This method known as electrolytic flocculation makes use of the surface charge of the microalgae to migrate it to an electrode of an opposite charge much like an electrolysis setup. The setup uses relatively lower amounts of energy and this has opened up doors to further research into scale up models for industrial applications (Poelman et al., 1997).

1.4.2 Centrifugation

Centrifugation is one of the most simple and straightforward methods of separating the algal biomass from the culture media (Harun et al., 2010). It is one of the most successful and most preferred techniques which can be applied when harvesting microalgae irrespective of the intended use of that biomass. Centrifugation is usually used after the initial dewatering step of flocculation to further remove the media that is contained in the

algal slurry just to produce and algal cake (De la Noue & Pauw, 1988). This method of harvesting algal biomass is very efficient at lab scale, but scaling it up to industrial scale production results in a rise in costs that make it an unfeasible technique to employ; this is because scaling up generally requires development of specialised centrifuges, such as continuous flow types which are very costly to setup (Grima et al., 2003). Other problems that could be associated with centrifugation of microalgae is the possible loss of cell cytosolic contents such as lipids and carbohydrates due to the high shear stress created by the centrifuge this is particularly observed in fragile strains and this stresses the need for optimisations of speed of centrifugation for each of the strains that would have been cultured (Heasman, 2000).

1.4.3 Filtration

Filtration is composed of many different types of setups which are characterised mainly based on the size of the membrane pores or the orientation of the porous membrane and the types that exist are: dead end filtration, microfiltration, ultra filtration, pressure filtration, vacuum filtration and tangential flow filtration(Zhang et al., 2010, Harun et al., 2010). Membrane separation is an important technique that has various applications in the biotechnology industry and it is seen as an attractive method that could have a wide variety of applications in the industrial production of microalgal biomass, particularly in recovery of the biomass and the metabolites of interest (Rossignol, 1999). The problems associated with membrane filtration are reduced performance due to membrane fouling over the filtration time(Kaghazchi, 2001). During the recovery of microbial biomass as time progressed there is an observed reduction in flux due to membrane fouling by cell debris and media contents, which leads to reduced recovery rates (Grima et al., 2003). There have been attempts to increase the recovery rates by increasing flux with membrane cleaning by air sparging and changing the membrane as it acquires some damage, but then the costs of maintaining a

high flux hence recovery rate have led to the increases in the cost of operation of the filtration system and this has made it a very expensive option for the use of microalgal biomass recovery (Zhang et al., 2010; Grima et al., 2003).

1.5 Other products from microalgae

The current technology of large scale production of microalgae is feasible, but the commercial aspect of the large scale production for biodiesel purposes is not economically feasible (Chisti, 2008). The problems with large scale production lie with the high cost of downstream processing of the culture, which can account for about 70 – 80% of the total cost of production (Grima et al., 2003).

In addition to lipids, microalgae also contain a varying level of proteins, carbohydrates, antioxidants and long chain polyunsaturated fatty acids such as EPA and DHA. Microalgae can be used for providing nutrients for human consumption, which is provided in the form of a powder generally as a food supplement (Brennan & Owende, 2010). It can also be used as animal feed and current applications in aquaculture make it a global industry (Borowitkza, 1997). Other applications such as in the nutraceutical industry for the production of products such as essential fatty acid supplements such as EPA and DHA make it an even more attractive as these are high value products (Harwood and Guschina, 2009).

The importance of Eicosapentaenoic acid (EPA, C20:5 w3) and docosahexaenoic acid (DHA, C22:6 w3) and arachidonic acid (AA, C20:4 x6) has been explored in the modern nutritional industry as some of the compounds that have multiple health benefits for people (Patil et al., 2007). Current dietary sources of these essential fatty acids are mainly from oilseed crops and marine organisms such as fish and shellfish (Mahaffey, 2004). Current commercial production of the w-3 and w-6 fatty acids has primarily been from marine fish, and it is thought that the increasing market demand will not be met by the declining fish

stocks, and therefore an alternative source must be sought after in order to meet these demands (Patil, 2007). Oil-seed crops such as sunflower and canola; and microalgae culture has been sought as a viable alternative source of EFAs. In comparison, microalgae have a much higher yield per unit area of land mass used and lipid productivity than crops, the relative number of inputs towards microalgae culture to produce the same amount of lipids as oil-seed crops is also considerably less and therefore making it a more attractive approach than using oil-seed crops(Schörken, 2009, Chisti, 2008).

There are complications with using commercially produced microalgae for human consumption. Currently the costs of producing microalgae biomass are very high, and this means that adding any products derived from microalgae will result in very expensive foods; the other problem is that there are fears of toxicological contamination that may cause more harm than good to human health (Mata et al., 2010). Most of the w-3 PUFAs that are in fish are derived from the marine food chain, and this has resulted in their use ubiquitous use in the aquaculture industry as feed additives, or fed to zoo plankton which is then used as live feed for the crustacean and finfish aquaculture industry (Patil et al.,2007; Mata et al., 2010). There are a number of microalgal species currently being used in aquaculture, pure strains of which are stored at the CSIRO algae culture collection (CSIRO, 2010). The most commonly used strains in the Australian aquaculture industry is Nannochloropsis, which is used in the production of rotifers and is found to have a lipid content ranging between 12 -53% of dry weight biomass and has high content of EPA (Mata et al., 2010; Roncarati et al., 2004); the other is Isochrysis sp.which has a lipid content between 7.1 – 33% of dry weight biomass and has a high content of DHA (Mata et al., 2010; Roncarati et al., 2004). Studies by Roncarati et al. (2004) have shown that these two species have high potential for large scale indoor culture and the composition of the

biomass can be improved by optimising the culture conditions, which will make it highly applicable to a hatchery practice.

1.6 Aims and objectives

The aim of the current study will be:

i. To use cultured/commercially available microalgae biomass as an alternative protein source for fish feed.

ii. To examine the palatability of microalgae as an ingredient in fish feed formulations.

iii. To observe any changes in the growth, performance and fillet quality of the yellowtail kingfish.

The aims of this study will be realised by using the commercially available microalgae species, *Spirulina* sp., known to have a high protein content (55 – 70% dw.) as an alternative source of protein in fish feed. Using this microalgae species in the feed formulations will provide an insight into formulating the proteins that will be derived from the two biorefinery microalgae species, *Nannochloropsis* sp. And *Isochrysis* sp. in fish feed. The palatability of the *Spirulina* sp. infused diet by Yellowtail Kingfish will be investigated by formulating different diets that contain different substitution levels of *Spirulina* for fish meal. The performance of the diets will be investigated by investigating how the diets affect the growth rates, physiology, and the flesh quality of the cultured YTK.

The study will evidence these aims through an examination of the status of commercial microalgae culture in South Australia, with a possibility of using available commercial producers as suppliers of microalgae for this project. This will have the potential of improving the client base of and product application of microalgae culture, providing future prospects for industry integration with the aquaculture industry.

2 Materials and Methods

The workflow of this study presented in Figure 1, indicates that the feed formulation process was a feedback loop system. The feed formulations were dependent on the amount of microalgae biomass that could be recovered and the nutrient composition of the microalgae biomass. Once the biomass was acquired and the feed formulations created, the tank setup was determined and the feed trial commenced and continued for eight weeks with daily monitoring and maintenance. Three diet formulations were created, one control and two microalgae substitution diets. Upon completion of the feed trial the fish were harvested and samples of fish were obtained from individual tanks and represented each dietary treatment. Dietary treatments were performed in triplicate, represented by three individual tanks for each diet formulation. The samples of fish obtained were analysed for fillet storage and diet performance with respect to its effect on the physiological properties of the fish.

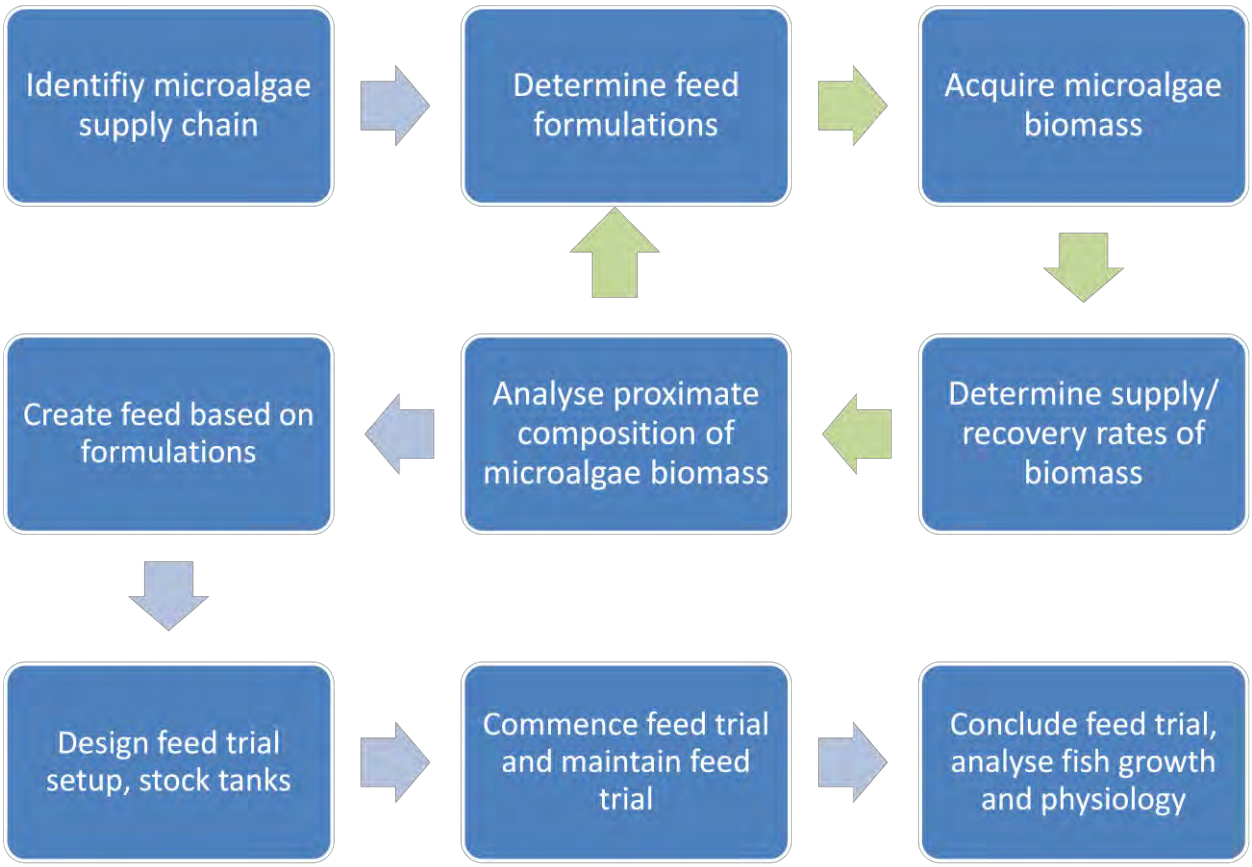

Figure 1: workflow process as excecuted for the microalgae feed trial.

2.1 Microalgae acquisition

Two species of microalgae namely *Nannoclhoropsis* sp. and *Isochrysis* sp. were cultured in outdoor 20m^2 raceway ponds and 4000L bioreactor respectively in f/2 media prepared in fresh sea water.

Nannoclhoropsis was harvested by collecting the culture media and adding an Fe^{3+} based flocculant, to allow the microalgae to flocculate, and the top layer of the water was removed and the algal slurry was dried in an oven at 55degC for 7days. *Isochrysis* was harvested by adding an Fe^{3+} based flocculant to 1000L of culture and it was left to flocculate overnight. The flocculant was collected and left to flocculate at 4°C for 4 days.

The flocculant was collected and centrifuged at 3000rpm for 5 min at room temperature. The resulting slurry was freeze dried in steel trays for 7days.

Due to a low recovery rate of the cultured microalgae, 15kg of dry *spirulina* powder was purchased from a commercial supplier at the rate of $\$70kg^{-1}$.

2.2 Microalgae analysis

For the nutritional information of microalgae it was imperative that the most accurate analytical methods were used. The Australian Measurement Institute was indentified to carry out the proximate analysis on the three species of microalgae namely the two biorefinery microalgae *Nannoclhoropsis* and *Isochrysis*, and the commercially available *Spirulina*. The proximate analysis was used to measure: total protein, fatty acid profile, total fat, fatty acid profile, carbohydrate, ash, and moisture content.

Table 1.

Proximate composition of microalgae as analysed by the national measurement institute

Microalgal sample	*Spirulina*	Isochrysis-nannochloropsis mix
Proximates ($g100g^{-1}$)		
Moisture	5.3	6.6
Protein (N x 6.25)	67.1	9.5
Fat (Mojonnier extraction)	4.6	8.4
Ash	6.2	56.5

2.3 Feed preparation

2.3.1 Feed formulation

Three diets containing two microalgae levels (20% and 30%) and one control were formulated using the standard Ridleys™ Aquafeeds YTK maintenance diet as a base Table 2. The diets were prepared using the cold pellet extrusion technique. The feed content and the microalgae inclusion levels were calculated and it was determined that from the raw ingredients to be used there would have to be several nutrient supplement that would have

to be added once the microalgae had been added to the feed. The microalgae included feed formulations were designed to closely match the nutritional content of the control feed as prepared by the feed manufacturer.

Table 2.
Ingredient formulation and proximate composition of experimental diets (as fed g100g^{-1})

Diet formulation	Control	Formulation 1	Formulation 2
Wheat 14	15.00	15.00	15.00
Lupins - Dehulled 35.0	10.00	10.00	10.00
Blood meal 93	8.00	8.00	8.00
Fish meal	40.00	20.00	10.00
Spirulina	0.00	20.00	30.00
Fish oil	6.82	7.51	7.85
Soybean meal	10.00	10.00	10.00
Corn gluten	8.00	8.00	8.00
Disodium phosphate	0.48	1.90	2.60
Methionine	0.02	0.00	0.06
Lysine	0.00	0.00	0.04
Taurine	0.04	0.21	0.30
RAP Finfish Vit/Min premix	0.30	0.30	0.30
Vitamin E 50 (Adsorbate)	0.04	0.04	0.04
total	100.08	100.32	100.43
Proximate composition (g100^{-1})[1]			
Moisture	9.62	6.90	5.44
Protein	50.63	50.66	50.73
Fat	12.00	12.00	12.00
Phosphorus	1.20	1.20	1.20
Amino Acids (g100^{-1})[1]			
Lysine	3.41	2.85	2.60
Methionine	1.13	0.97	0.95
Cysteine	0.61	0.48	0.41
Meth + Cyst	1.73	1.44	1.36
Threonine	2.03	1.96	1.92
Leucine	4.56	4.46	4.41
Isoleucine	1.99	1.95	1.94
Tryptophan	0.53	0.38	0.31
Arginine	2.90	2.88	2.87
Valine	2.78	2.73	2.71
Phenyl	2.41	6.79	8.98
Histidine	1.70	2.96	3.58
Glycine	3.04	2.44	2.15
Proline	1.08	0.94	0.87
Tyrosine	1.44	1.43	1.42
Aspartic	2.48	2.23	2.11
Glutamine	3.36	3.20	3.12

Alanine	1.72	1.61	1.55
Serine	1.14	1.06	1.03
Taurine	0.40	0.40	0.40

[1]Calculated values

2.3.2 Feed extrusion

Raw ingredients of the feed were obtained from the control diet manufacturer, Ridleys Aquafeeds. Three feed formulations were prepared according to Table 2 above, formulation 1 is the control; formulation 2 is diet 1, which has a 20% *spirulina* substitution for fish meal, the main protein source in the diet; and formulation 3, is diet 2, which has a 30% *spirulina* substitution for fish meal.

Preparation of the feed pellets was carried out in 5kg batches due to the limitation of the extruder and the drying ovens. The dry ingredients were weighed out using an electronic mass balance and mixed for 5 min with a Q60 Hobart bread dough mixer, and were mixed for 5 min at low speed. Next the fish oil was weighed out and with the mixer set to 5min mixing at low speed the fish oil was slowly added to completion. Reverse Osmosis (RO) water was added at 20% (v/w) of the total weight of the feed and mixing was continued until the mix had the right consistency for extrusion.

The feed mix was transferred to the extruder and was extruded and cut to pellet sizes of approximately 10mm. the extruded pellets were placed on perforated trays, and placed in a drying oven set to 55°C for 17h.

The dried pellets were sampled and the moisture content was tested by drying the samples of the pellet in an oven at 105°C for 2h. The weight difference of the pellets was used to determine the moisture content, which was required to be less than 10% (w/w) for long term storage. The dried pellets were placed in bags, sealed and stored at 4°C until they were needed for use.

2.4 Yellowtail Kingfish feed trials

2.4.1 Culture system

Prior to stocking the tanks, they were prepared by setting the flow rates of the recirculated sea water system to a flow rate of six litres every 15seconds using a graduated bucket; the resulting circulation flow rate was $0.4Ls^{-1}$. The water quality parameters, such as temperature, pH and dissolved oxygen were measured daily prior to feeding, and the salinity was measured weekly. The tanks were filled with filtered sea water, and air stones were used to provide continuous aeration.

2.4.2 Fish

Sub-Adult Yellowtail Kingfish (*Seriola lalandi*) were stocked from a batch obtained from SARDI Aquatic Sciences West Beach, Adelaide by first anaesthetizing the fish in AQUI-S(r) anaetshetic (iso-eugenol) at a concentration of $0.0714mlL^{-1}(v/v)$ for 2min, and were weighed and placed in 1000L fiberglass tanks filled with filtered sea water. To maintain a uniform initial biomass in each tank, a stocking sheet was prepared in excel, which calculated the biomasss in each tank and the difference between the highest and lowest biomass in each tank. The fish were stocked in a manner that maintained as minimal a difference as possible in biomass between the tanks.

2.4.3 Feeding regime

Feeding the fish was carried out once daily commencing at 10.30am for 6 days, and the fish were starved on one day of the week for 24h to allow the fish to build appetite. Feeding was carried out by hand and each tank was fed for approximately 10 minutes to apparent satiation. Apparent satiation was determined by 2 succesive pellets sinking to the bottom of the tank. The feeding trial was maintained for 8 weeks, between the months of August and

September, where temperatures ranged between 11 – 16 °C as the seasons were changing from winter to spring. Prior to the conclusion of the feed trial, the fish were starved for 48hrs to allow for their digestive system to empty out all the food in their gut. At the end of the study the fish were anaesthetized with AQUI-S(r) anaetshetic (iso-eugenol) at a concentration of, and killed with a spike through the brain. The body weight of each fish and the length was measured, and the fish were immediately placed in an ice-box. In each tank, a representative sample made up of 5 fish was collected and stored in Styrofoam cooler boxes packed with ice, where storage fillet quality and lipid oxidation analysis of each fish would be carried out at the Port Lincoln Marine Science centre. The remaining fish were stored in a -20 °C cold room until they would be needed for further analysis.

2.5 Fish analysis

The sample of 5 fish collected from each tank was packed in a Poly box packed with ice, sealed and was transported to the Port Lincoln Marine Science Centre for analysis. Approximately 48 h after harvesting, each fish was filleted and gutted, and the fillet, viscera, and liver weight measured to determine the fillet yield, viscerosomatic index (VSI) and the hepatosomatic index (HSI) respectively. The fillets were stored in zipper bags at 4°C, in a constant temperature room.

2.6 Colour analysis

The fillet colour analysis was carried out as per method developed at the PLMSC. Digital colour photographs were taken of the left side fillets under controlled light conditions consisting of a light diffusing box (cocoon 70 Lighting tent) under two 2x500Watt Summit halogen flood lamps. The digital camera (Canon Powershot G9) was white balanced with a fixed aperture, shutter speed, zoom and sensitivity. The digital photographs were taken at day 0, 2, 4, and 8 post-filleting; following which the photos were transferred to Photoshop ™. In this program the 'blur average' function was applied to a prescribed area of the fillet

(dorsal area) to yield a quantitative average colour value represented by the L*a*b* coordinated from the program. The individual blurred areas where then pooled within the treatment groups of the fish fed a particular diet and a blur average was applied to that pool to determine the average colour coordinates for each feed treatment. Colour separation between the treatments was determined by plotting a graph of the quantitative average colour values from each treatment on a 3D chart, and this was used to determine whether there would be a difference in the colour of the fillets fed the different diets.

2.7 Lipid oxidation

Tissue samples from the right side fillets of each fish were analysed by Thiobarbituric acid reactive substances (TBARS) assay by obtaining flesh samples using a coring tool at days 0, 2, 4, and 8 post-filleting, corresponding with the colour analysis photography on the same area where the blur average was prescribed. Tissue samples were then stored in a -80°C refrigerator until required for analysis. Upon analysis frozen samples were crushed with a ceramic mortar and pestle on a bed of dry ice. Approximately 1g of the powdered sample was then added to 5mL of 0.6M Perclhoric Acid (PCA), placed on ice for 20min and centrifuged at 4000rpm for 5min at 4°C. The supernatant obtained was frozen in cryo-vials at -80°C until needed for assay. 500µL of thawed supernatant was then incubated at a 1:1 (v/v) ratio of the thiobarbituric acid (TBA) reagent at 100°C for 35mins and then allowed to cool at room temperature. Colour development was measured using a Thermo Labsystems, Multiskan Ascent 96well plate reader with a 540nm filter. The level of lipid oxidation was quantified against a known standard curve.

2.8 Statistical analysis

All the statistical analysis for comparison of means was carried out using a one factor ANOVA, with the diet fed being the factor of analysis. This method was suitable as it provided a comparison of means against a known factor that was a categorical variable.

Post Hoc comparisons chosen for this study were the Student-Newman-Keuls tests as it allowed for comparisons between across the multiple groups.

3 Results

Due to insufficient biomass recovery, a different species of microalgae was purchased from a different commercial supplier of microalgae. The results presented reflect the acceptance and performance of *Spirulina* as formulated in the fish feed. Fish were fed for eight weeks, and observations made during the first four weeks indicated that the fish exhibited lethargic swimming patterns, characterized as winter syndrome (Miegel *et al.*, 2010). The fish quickly adapted as swimming patterns returned to normal and feeding was very active.

3.1 Microalgae proximate composition

Three species of microalgae were analysed for proximate composition, the services were rendered by the National Measurement Institute in Melbourne Victoria. Two of the microalgae species that were analysed were made into a mixture that comprised the *Nannochloropsis* sp. and *Isochrysis* sp. mixture in a 1:1 ratio. The third species of microalgae was obtained from a vegetarian health food supplement store, which was *Spirulina* sp. and it was sold under the brand name Oxymin® *Spirulina* Pure, Organically grown; which is used a human vegetarian protein supplement. The protein content of the *Spirulina* was analysed to be 67.1g100g^{-1} dry biomass, compared to the 9.5g100g-^{1} dry biomass for the *Nannochloropsis* and *Isochrysis* mixture which had most of its biomass composed as ash at 56.5g100g^{-1} dry biomass.

3.2 Diet formulations

Three isonitrogenous (50% crude protein) diets were prepared. *Spirulina* replaced 0, 50% and 75% of fish meal in the three diet formulations. Several assumptions were made during the diet formulation, cysteine, Phosporus and Taurine was not analysed for in the microalgae, and was, therefore assumed for each formulation based on the content of the rest of the ingredients.

3.3 Growth and feed utilisation

During the 8 week feed trial the water temperature in the tanks ranged from 11.4°C – 16.3°C, pH remained in the range 7.98 – 9.78, and the dissolved oxygen was always above the level of 6.9mg/L.

The data summarized by Table 3 indicates that fish fed the control diet had the lowest survival rate of 86.1% as compared to the fish fed the 20% *spirulina* diet with the highest survival rate of 94.4% followed by the fish fed the 30% *spirulina* diet with a survival of 89.9%.

Yellowtail Kingfish fed the three different diets actually lost weight and condition throughout the experiment. According to the results summarized by Table 3, the initial individual weight of the fish at the start of the study was not statistically different between the tanks. The condition index of the fish prior to stocking the tanks, which was based on a sample of 20 fish, was 0.152 $\pm$0.003. The initial weights of the fish amongst the treatment groups were very similar with the lightest fish measured to weigh 742.3g and the largest fish weighing 750.0g. At the end of the feed trial the results show that the average fish weight amongst the treatment groups was not vastly different. The statistical analyses show that there was insufficient evidence to show a statistically significant difference ($P > 0.05$) between the individual fish weight for the fish fed the 3 different diets over the feed trial period.

The specific growth rates according to Table 3were negative, and this data was based on the differences between the average fish weight at the end of the feed trial and the average fish weight at the start of the feed trial. The final adjusted biomass per tank shows minimal difference between the dietary treatment groups and the differences between the groups were not statistically significant ($P > 0.05$). The adjusted biomass gain and feed conversion ratio for all tanks fed the different diet formulations was negative, but there was

insufficient evidence to prove a statistically significant difference ($P > 0.05$) between the three diet formulations.

The fish condition deteriorated during the feed trial period, which is evident when comparing the condition indices of the initial sample of fish prior to stocking the feed trial with the fish after the eight week feed trial. There is no statistically significant difference ($P > 0.05$) between the condition indices of the fish fed the three diet formulations. The body condition of the fish was significantly affected by feeding the 30% *spirulina* diet. The final Hepatosomatic index (HSI) and the Viscerosomatic index (VSI) of the fish fed the control and the 20% *spirulina* diet were not statistically different between the two dietary formulations ($P > 0.05$), but the final HSI and VSI of the fish fed the 30% *spirulina* diet was significantly lower ($P < 0.05$) than of the fish fed the control and 20% *spirulina* diets. The fillet yield of the fish was found to be affected by the diet formulations and according to Table 1 the fish fed the control diet had a significantly higher ($P < 0.05$) fillet yield than the fish fed the 20% and 30% *spirulina* diet.

The total feed intake for the three diet formulations varied with the substitution level of *spirulina*. According to Figure 2, the apparent cumulative feed consumption of the diet formulations were similar in the first week of the feed trial, and from the second week, the differences between different diets became more apparent as the feed trial progressed. Figure 3 shows the total feed consumed during the feed trial period, and it shows a decreased total feed consumption with an increased *spirulina* substitution level. There was a significant difference ($P < 0.05$) in total apparent feed consumed between the control and the highest *spirulina* substitution level.

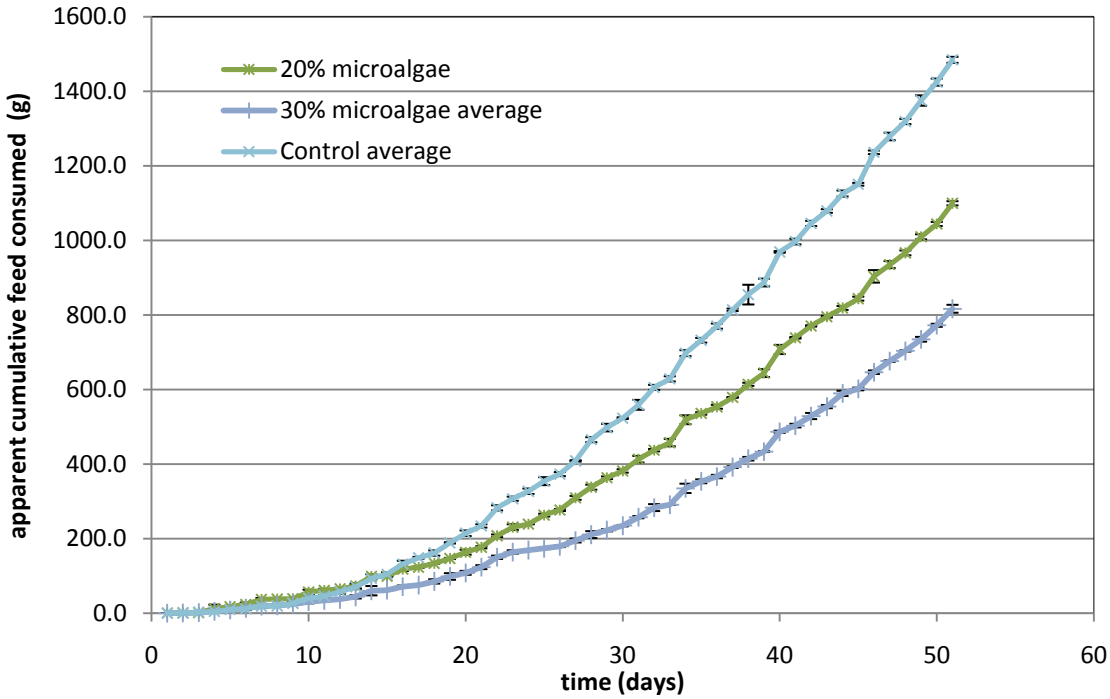

Figure 2: apparent cumulative feed consumption over the feeding period for the three diet formulations

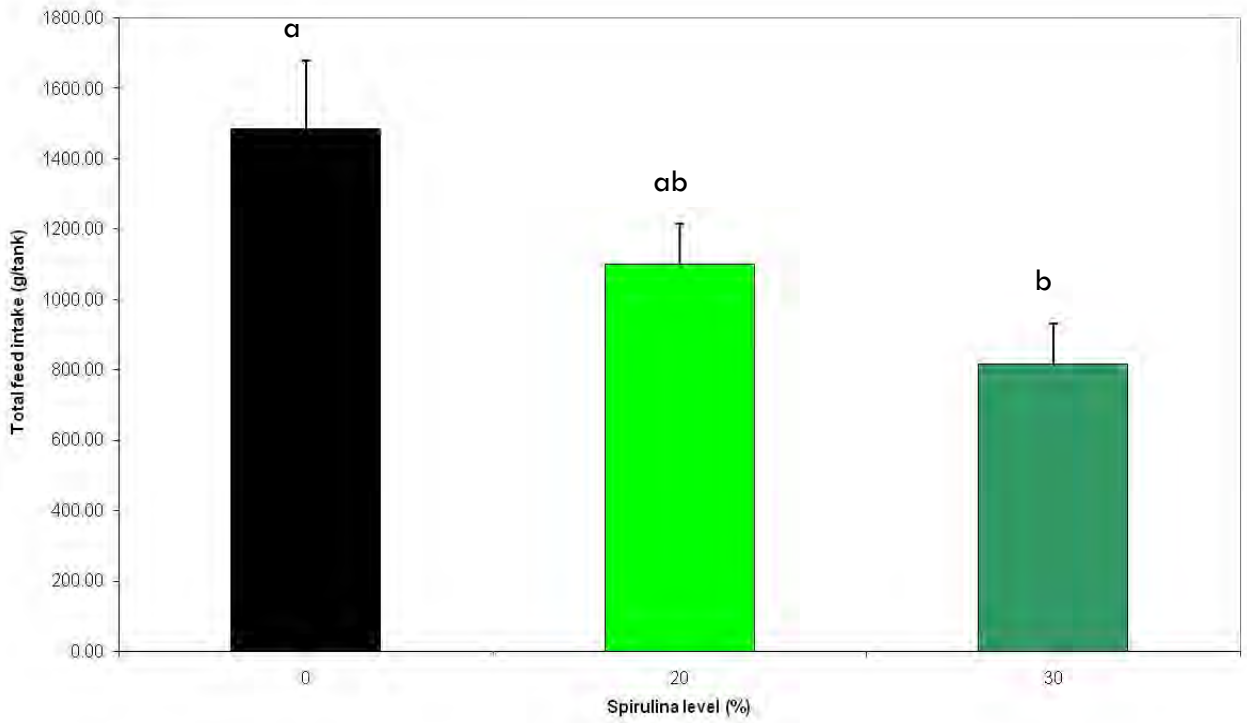

Figure 3: total apparent feed intake for each dietary formulation[1].

[1]bars that share the same script are not significantly different (One-Factor ANOVA, SNK, $P>0.05$).

Table 3: Results of one-factor ANOVA on measured growth parameters of yellowtail kingfish fed three different dietary treatments[1]

Measured parameters	control	20% *spirulina*	30% *spirulina*	*P*-value
Survival (%)	86.1	94.4	88.9	*n/a*
Initial individual weight (g/fish)	748.2 ±22.8[a]	750.4 ±17.9[a]	742.3 ±22.1[a]	0.544
Final individual weight (g/fish)	747.5 ±23.9[a]	654.5 ±19.4[a]	683.1 ±17.9[a]	0.072
Individual weight gain (g/fish)	-0.7 ±1.1	-65.9 ±1.5	-59.2 ±4.2	*n/a*
SGR (% day^{-1})	0.000 ±0.011	-0.010 ±0.015	-0.019 ±0.042	*n/a*
Initial biomass (g/tank)	8905.6 ±186.4	8805.2 ±62.7	9814.1 ±17.6	0.127
Final adjusted biomass (g/tank)	9108.8 ±441.7	8143.6 ±173.7	8070.9 ±213.7	0.574
Adjusted biomass gain (g/tank)[2]	1587.7±540.3	-275.8 ±188.9	-328.3 ±632.3	
Apparent feed intake (g/tank)	1484.2±195.9[a]	1099.2 ±116.3[a]	816.5 ±66.1[b]	0.037
Apparent FCR	-4.02	-1.52	-0.74	
Condition index (initial = 0.152 ±0.003)	0.142 ±0.002	0.133 ±0.002	0.131 ±0.005	0.264
Final HSI (%)(initial =	0.804 ±0.08[a]	0.808 ±0.04[a]	0.562 ±0.05[b]	0.009
Final VSI (%)	4.61 ±0.13[a]	4.48 ±0.16[a]	4.10 ±0.11[b]	0.026
Fillet yield (g/fish)	365.9 ±16.4[a]	298.6 ±9.4[b]	303.4 ±15.3[b]	0.02

[1] Values are means (± standard error) of n = 3 replicate tanks for each dietary treatment; Values that share the same superscript are not significantly different (One-Factor ANOVA, SNK, *P*>0.05).

[2] Adjusted biomass gain = (Final tank biomass + mortality weight) – weight of initial tank biomass

3.4 Tristimulus colour analysis

The raw fillets stored for eight days at 4°C showed a shift in the tristimulus colour characteristics values over the storage period. The L- values (lightness) were similar after zero days post filleting for the entire sample set Figure 4. Though the a - values (redness) were similar for the fish fed the included *spirulina* diets, the fillets for fish fed the control diet were slightly redder throughout the eight days refrigerated storage period. This quantitative assessment was also supported by the qualitative assessment conducted by the Cleanseas Product Quality Manager. The reduction in fillet redness over time appeared to be slower in fillets from fish fed the control diet and were significantly redder ($P < 0.05$) after eight days storage compared to those that were supplemented with *spirulina*. There appeared to be no differences in fillet b- values (yellowness) at day zero between the dietary treatments, however the increase in fillet yellowness was slower in fish fed the 30% *spirulina*. At day eight post filleting fish fed the control and 20% *spirulina* diet had clearly elevated b- values that were significantly ($P < 0.05$) lower when compared to those fed the 30% *spirulina* inclusion diet Figure 5.

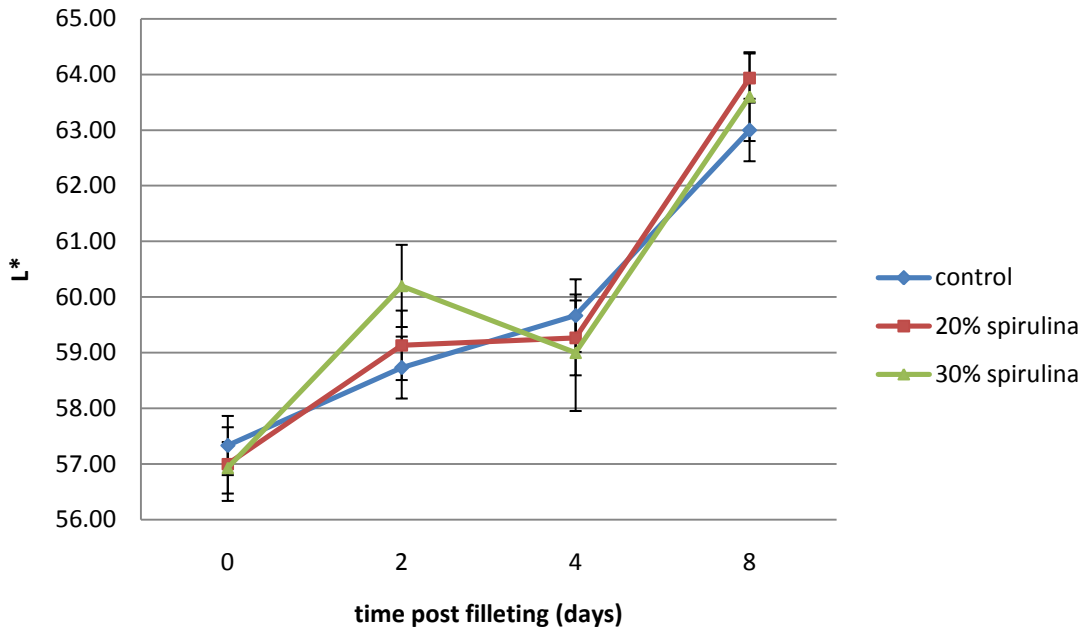

Figure 4: Change in fillet L* (lightness) values during the 8 days refrigerated storage for fish fed diets the control, 20% *spirulina* and 30% *spirulina* diets.

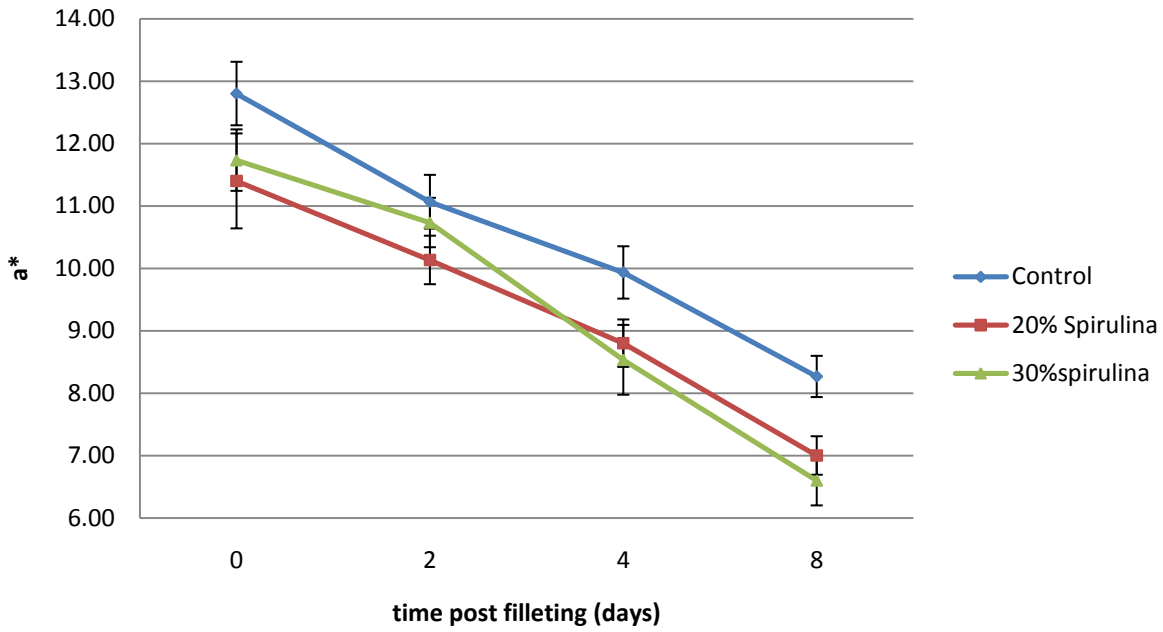

Figure 5: Change in fillet a* (redness) values during the 8 days refrigerated storage for fish fed diets the control, 20% *spirulina* and 30% *spirulina* diets.

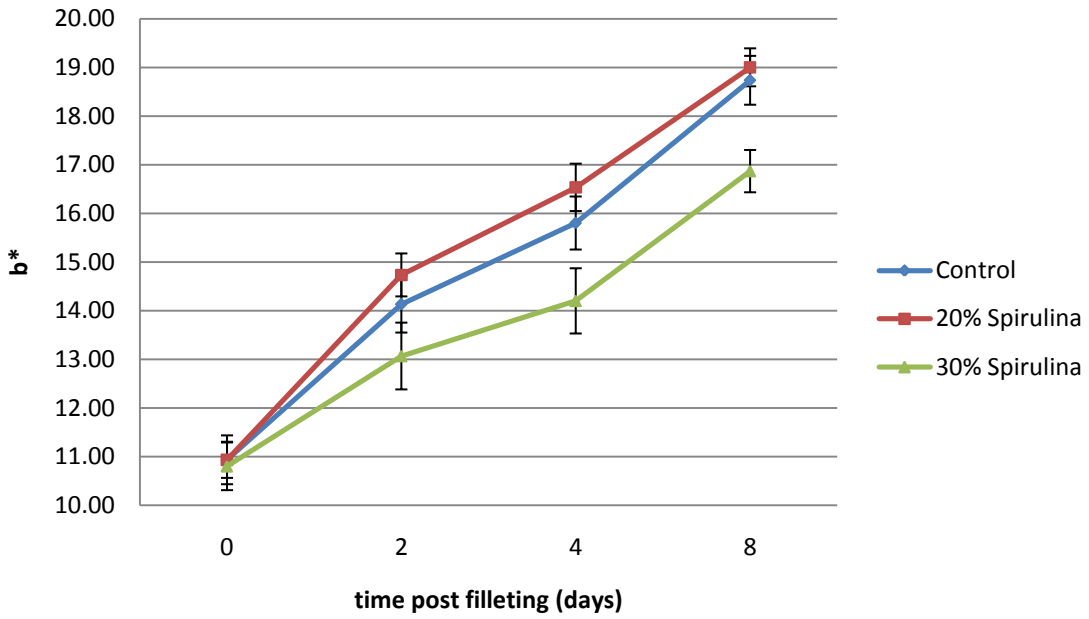

Figure 6: Change in fillet b* (yellowness) values during the 8 days refrigerated storage for fish fed diets the control, 20% *spirulina* and 30% *spirulina* diets.

The individual blurred areas of the tristimulus colour parameters from the treatment tanks were pooled Figure 6 into treatment groups and another "blur average" function applied to the pool revealed visual differences between the fillets of different dietary treatments Figure 7.

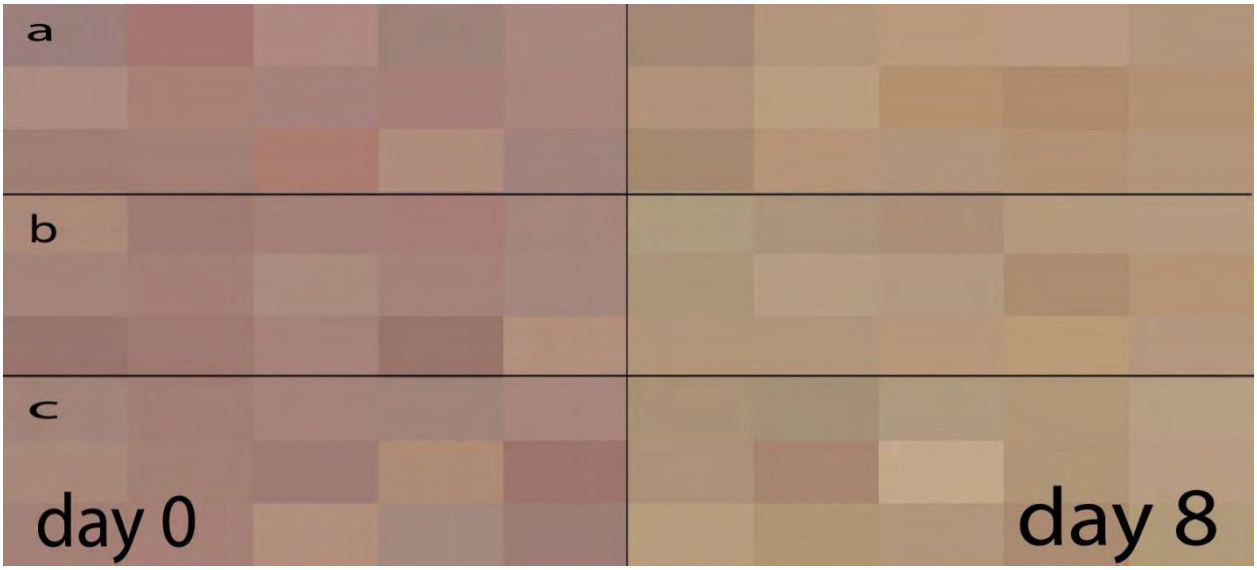

Figure 7 : Blur averages from 15 individual fillets in each diet treatment. (a) pooled samples of the control treatment at days 0 and 8; n=15 (b) pooled samples for the 20% *spirulina* treatment at days 0 and 8; n=15 (c) pooled samples for the 30% *spirulina* treatment at days 0 and 8; n=15

Figure 8 : Pooled blur averages from 15 individual fillets in each diet treatment. (a) represents blurred samples of the control treatment at days 0 and 8 (b) represents blurred samples for the 20% *spirulina* treatment at days 0 and 8 (c) represents blurred samples for the 30% *spirulina* treatment at days 0 and 8

The "blur averages" in Figure 7 provide a visual confirmation of the differences between the fillet colour for the individual treatments between day zero and day eight.

Figure 8 provides a visual confirmation of the colour separation between the fillets of the fish given the dietary treatments. There were no observable differences between the individual dietary treatments on day 0 and day 8 refrigerated storage.

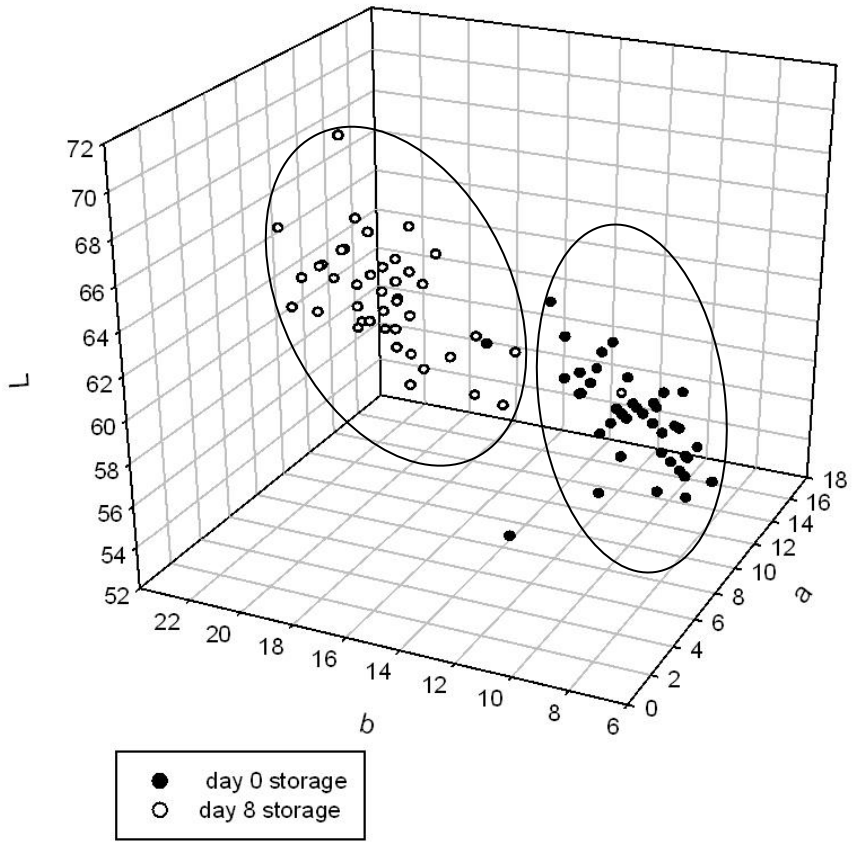

Figure 9: L*a* and b* colour space values of YTK fillets after applying a Photoshop™ "Blur Average' to the dorsal sampling site of fillets fed the control, 20% *Spirulina*, and 30% *spirulina* diets at Day 0 and day 8 refrigerated storage.

3.5 Lipid oxidation

The level of lipid oxidation (TBARS thiobarbituric acid reactive substances) was found to be insignificantly higher ($P > 0.05$) in fillets from fish fed the 30% *spirulina* diet compared to samples fed the control and 20% *spirulina* diets after day 8 refrigerated storage but found to be insignificantly different on the other days. The levels of TBARS from fillets fed the control diet and 20% *spirulina* diet were comparable and not different from each other.

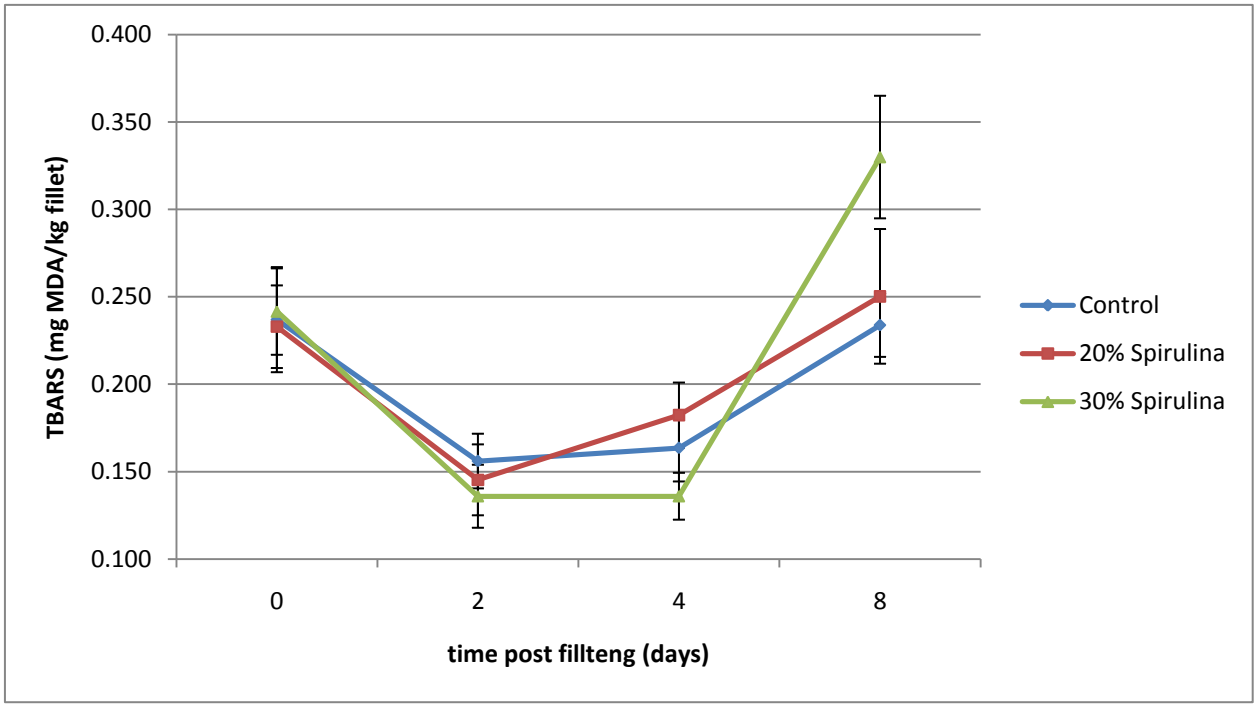

Figure 10: Changes in TBARS (lipid oxidation) concentrations within the fillets from fish fed the control, 20% *spirulina*, and 30% *spirulina* diets during 8 refrigerated; n=15

4 Discussion

4.1 Microalgae supply

In the current study, notable observations that could be made are that there is currently a very limited commercial supply of microalgae in South Australia. This could be attributable to the limited development of infrastructure for large scale commercial culture of microalgae, which is an industry still in its infancy stage. The development of microalgae has only just recently received attention, with most of the major projects having been started in the past five years. There are a few initiatives that have been implemented in Australia, amongst them the collaboration between the South Australian Reasearch and Development Institute (SARDI) and Australian Renewable Fuels (ARF) for the development of a photobioreactor culture of saline water microalgae(Kirke, 2006).

Growth and progress in the microalgae culture industry has largely been limited by the economics of the large scale culture of microalgae, whereby any increases in culture volumes lead to a disproportionately higher increase in overall costs of the operation (Knuckley *et al,* 2006; Brennan & Ownede, 2010). This has made single product large scale microalgae culture an economically unattractive venture, and has thus limited it towards the production of high value products such as human food supplements, nutraceuticals, and feeds for live rotifers for use in various aquaculture industries (Patil *et al.,* 2007; Chisti, 2008 and Harwood & Guschina, 2009). A significant contributing factor towards the large scale culture of microalgae is cost of harvesting and downstream processing of the microalgae biomass from the culture media, which can comprise up to 80% of the total production cost (Grima et al, 2003; Knuckley *et al,* 2006). Focus is now shifting towards a biorefinery approach of microalgae culture, which will provide options that make the large scale culture of microalgae more economically feasible, and aim to provide a zero waste scenario from

the commercial production of biofuels from microalgae (Griffiths, 2009, Harun et al., 2010, Brennan and Owende, 2010).

Cultures of the biorefinery microalgae carried out in outdoor systems where *Nannochloropsis* was cultured in a raceway pond, and *Isochrysis* was cultured in a closed tubular photobioreactor provided very low yields. The limitation was mainly attributed to large fluctuations in weather conditions such as temperature and available sunlight. On certain days the weather conditions were moderate with clear skies and moderate average temperatures of 22°C. Extreme conditions on other days would be average temperatures of 33°C with high light intensities due to absence of cloud cover, or low average temperatures of 15°C and low light conditions created by heavy cloud cover. Times of high light intensity and high temperatures significantly slow the growth rates of microalgae; this is because optimal growth of microalgae occurs at temperatures between 20 and 25 °C (Converti et al., 2009) and light intensities provided by moderate cloud cover(Janssen, 2003). Nutrient productivity is also limited in microalgae cultured in conditions that are not optimal for their growth and several studies have observed decreased lipid productivity and a decreased nitrogen composition of *Nannochloropsis* and *Isochrysis* growing in less than optimal conditions in outdoor cultures (Rodolfi, 2009, Converti, 2009, Vergani, 2008). These observations are consistent with what was observed on microalgae cultured during the months of March and April 2010 at PIRSA-SARDI, and according to the results in Table 1, chapter 2 the ash content in the *Nannochloropsis* and *Isochryis* of 56.5% could be an indicator of non-optimal culture conditions of the microalgae. Alternative sources of microalgae biomass were investigated throughout the country and there was a need to identify a supplier of good quality microalgae biomass.

Current observations on the supply of microalgae in Australia are mostly limited towards use in the aquaculture industry with most products being sold as algal slurry concentrates, which consumers use to subculture their own stock for use in hatcheries. An example of such a product, which was sold under the name Rotifer Diet™ which contained a mixture of *Nannochloropsis* and *Tetraselmis*, was sold at a cost of A94.00L^{-1}$ and had a dry biomass yield of about 20gL^{-1}. Another product was sold as a human nutrition supplement sold under the brand name Oxymin™ Pure Organically grown *Spirulina*, which was made up of 100% dry *Spirulina* biomass, and was sold at a rate of A60kg^{-1}$, but it can be sold for as low as A42kg^{-1}$ for large quantities.

4.2 Microalgae feed trial

The feed formulations in this study were created to investigate the feasibility of using microalgae derived proteins in Yellowtail Kingfish (*Seriola lalandi*) feed. This was carried out by comparing the acceptance, effect on growth rates, body composition and condition of *S. lalandi* fed the *Spirulina* formulated diet and *S.lalandi* fed the control diet.

According to the feed consumption data the most feed consumed over the eight week period was the control diet with a total of 1484.2 ±195.9g followed by the 20% *spirulina* diet with a total of 1099.2±116.3g and lastly 30% *spirulina* diet with a total of 816.5 ±66.1. The trends shown by corresponding graph indicate that during the first week of the feed trial, all three diets were accepted to the same degree, but the apparent differences observed in feeding rates thereafter may suggest preference for the feed that contains less *Sprulina*. The obvious differences that were observed between the different diet formulations were their colour and smell. The diet with the *spirulina* substitution had a strong smell, similar to that of

plant leaves and the control diet had a stronger fish smell in comparison, and this may well be one of the contributing factors to the acceptability of the diets by the fish. There is limited information that has been published regarding the food and feeding habits of *S.lalandi* however, studies by Vergani *et al,* (2007) have investigated the contents of adult *S.lalandi* in South America and were found to comprise mostly *Trachurus lathami*, a small pelagic schooling fish. Similar observations have been made to confirm these findings on other parts of the globe such as New Zealand and Australia (Poortenaar *et al.,* 2001), and this confirms the preference of *S.lalandi* for feed that closely resembles the taste characteristics of its prey. Suggestions for future studies that use microalgae in feed for *S.lalandi* would be to design feeds that do not have a vastly different taste and smell from feeds that contain a large proportion of fish meal, as observations in this study have shown a preference for feed that had higher fish meal content.

The survival of the fish during the feed trial period was 86.1% for fish fed the control diet, 94.4% for fish fed the 20% *spirulina* diet, and 88.9% for fish fed the 30% *spirulina* diet. Most of the mortalities occurred during the first four weeks of the feed trial period. Water temperatures during the study were lower than the required optimal temperatures for culture of YTK, at the commencement of the feed trial the average temperature was on average 12°C, and changed by an average of 1.5°C during the first four weeks to an average of 13.5°C. During this time period the swimming pattern of the fish was lethargic, which is characteristic of the winter syndrome as described by Sheppard (2004) to be caused by low gut motility due to low water temperatures. The anecdotal evidence presented above provides a basis for attributing the mortalities observed during the feed trial to be most likely caused by a combination of stress imposed on the fish during the stocking procedure and the low water temperatures.

The final individual weight of the fish was less than the initial individual weight of the fish for all the fish fed the three diets; the differences between the three dietary treatments were however, insignificant. The specific growth rate of the fish was negative for the fish fed the three diets, and the differences between the diet formulations were insignificant. The final adjusted biomass showed an increase in biomass of 1587 ±540.3g for fish fed the control diet and a change in biomass of -275.8 ±188.9g and -328.3 ±632.3g for fish fed the 20% *spirulina* and 30% *spirulina* diets respectively. The reduction in biomass indicates that the fish consumed the diets well below their maintanence levels, and have resorted to using their fat stores to acquire energy to maintain their daily metabolism.

Fish fed the three diets lost condition from 0.0152 ±0.003 prior to stocking the tanks to 0.142 ±0.002 in fish fed the control diet, 0.133 ±0.002 in fish fed the 20% *spirulina* diet, and 0.131 ±0.005 in fish fed the 30% *spirulina* diet. There were no significant differences between the three diets on the final condition index. The effect of the three diet formulations on the hepatosomatic index (HSI) and the viscerosomatic index (VSI) during the feed trial was also negative, which meant that the liver decreased in size and the fish lost visceral fat during the feed trial. The findings in this study are consistent with findings by Hamre *et al.,* (2003) who observed decreases in dry matter and lipid content of Norwegian spring-spawning herring (C*lupea harengus* L), during the winter and spawning migration periods.

The decrease in feed intake of *S.lalandi* in the winter period is due to a decrease in the transit time of the feed through the gut of the fish (Miegel *et al.,* 2010)(unpublished). Studies by Watanabe (2001) indicated that feeding rates of *Seriola quinqueradiata* decreased significantly in winter, with required feeding rates of up to three times a week. Increases in feeding rates did not result in any further increases in biomass gain by the fish, and chemical analyses of the intestine and the digesta have shown that at low water temperatures, the

feed was partially digested and in the stomach and remained there for up to 36 h (Watanabe *et al.,* 2001). These findings were confirmed by Miegel *et al.,* (2010) who confirmed that the gut transit time of feed through the digestive tract of *S.lalandi* correlated with water temperature and noted that shorter transit times were observed during higher temperatures between 23 and 28 °C. He further stated that the gut transit times were not influenced by the feeding rates, as the fish simply did not accept the diet.

4.3 Fillet Quality

The effect of *Spirulina* on the fillet quality of the fish was investigated using tristimulus colour analysis and TBARS assay. The tristimulus colour analysis was carried out to investigate changes in the appearance of the fillet and thiobatituric acid reactive substances (TBARS) to further quantify changes in the lipid oxidation levels, which affect fillet colour, over short term storage of eight days under refrigerated conditions maintained at a temperature of 4°C.

4.3.1 Tristimulus colour analysis

This is a method that involves the use of digital photography to assess the quality of fish fillets and it was developed at the Port Lincoln Marine Science Centre. The colour analysis makes use of the *CIE Lab* colour space to characterise the colour content of a digital image. The *Lab* colour coordinate system provides a perceptually uniform base for the comparison of different digital colour images; any differences in the colour space are approximately equal to the differences observed by the human eye (Liapis S., unkown). The sensitivity of the human eye to colour differences is not always uniform, and this is due to variations in the parameters surrounding perception of the object. Parameters such as distance from the

object, background colour, light and spatial pattern constantly cause the human perception of the image to change as a response of the adaptation by the eye to provide as clear an image as possible (Zhang X, 1997). To maintain a high precision of the quantified colour parameters in this assessment, it was imperative to have minimal variations in light distribution and intensity, camera sensitivity, and distance from subject when photographing the different fillets.

The Lightness (L* value) parameter describes the lightness of the raw fillet and is distinct and independent of the chromacity coordinates (*a,b*) (Liatis and Tziritas, 2004). The L* values of the fillets during eight days refrigerated storage increased in the fillets from the fish fed the three diet formulations. The fillets from the fish that were fed the control diet had higher L* values than the fillets from the fish that were fed the 20% *Spirulina* and 30% *spirulina* diet but the difference was statistically insignificant. These observations on the change in L* values are consistent with previous studies performed on different species of fish, which have shown L* values to generally increase in fillets that have been stored in refrigerated conditions over a period of time (Schubring, 2003) Jensen *et al.,* 1998). The slightly higher L* values from the fish fed the control diet could be attributed to a slightly higher fat content in the fillets. Although this has not been tested, studies by Jensen *et al*. (1998) have shown that fillets from rainbow trout (Oncorhynchus mykiss) that were fed feed with high fat content has significantly higher L* values than fillets from the fish that was fed diets with a lower fat content. This evidence could be applied to our study as the fish that were fed the *Spirulina* formulated diets had consumed less feed than the fish fed the control diet and were thus more likely to have fillets with a lower fat content. The change in L* values of the fillets could be attributable to the change in the protein structure in the muscle which has

lead to a change in the light scatter and absorption properties of the flesh (Einen *et al.,* 1998).

The redness (a* value) describes the red colour that is present in red meat is due to the protein mygloblin (myb), an oxygen binding protein found in muscle fibres. *S.lalandi* fillets contain very little myb but extensive reviews by Faustman *et al* (2010) have shown that myb oxidation could be a facilitator of lipid oxidation and vice versa. Fillets from the fish in this study showed a decline in a* values during the eight day storage, this is consistent with observations made in similar studies which show that a* values decline over time in fillets stored under refrigerated conditions (Schubring *et al.,* 2003; Jensen *et al.,* 1998). The decline in a* values was observed in the fillets fed the three different diet formulations could be explained to be primarily lipid oxidation of the polyunsaturated fatty acids present in the fillets (Jensen *et al.,* 1998). This correlates with observations made on the lipid oxidation in the fillets that were from fish fed the three dietary treatments .Comparing the data in lipid oxidation levels and redness levels, on day 8 the fillets from fish fed the 30% *spirulina* diet had the highest lipid oxidation levels, and the lowest a* values followed by fillets from fish fed the 20% *Spirulina* diet, with the median oxidation levels and median a* values and lastly the fillets from fish fed the control diet with the lowest lipid oxidation levels and the highest a* values in comparison.

The yellowness (b* value) describes the yellow that is present in the fillets, and this colour may come from fats within the fillets or various muscle proteins that comprise the fillet (Robb *et al.,* 2000). The fillets from the fish fed the three diets exhibited an increase in the b* value, and is consistent with observations made in other studies (Schubring *et al.,* 2003; Jensen *et al.,* 1998). The change increase in the b* value could be due to the increase in oxidised polyunsaturated fatty acids or heam proteins present in the fillets from fish fed the

control diet and the 20% *spirulina* diet had higher b* values as compared to fillets from fish fed the 30% *spirulina* diet. This could be a due to the high feed consumption by the fish fed the control and 20% *spirulina* diets which have resulted in fish with higher polyunsaturated fatty acids. Muscle pH during fish slaughter could have also contributed to the higher b* values, which would be caused by anaerobic killing of the fish resulting in a build up of lactic acid within the muscle, leading to a drop in muscle pH and thus changing the protein conformation within the muscles (Robb *et al.,*2000).

4.3.1.1 Visual differences

In this experiment, colour swatches were generated by pooling individual "blur averages" to determine any visual differences between the treatment at the beginning and at the end of the storage period. According to the colour swatches generated, there was a difference in the fillet colour between day 0 and day 8 of fillet storage across all the fillets fed the different diet formulations. There is however, no visual difference between the treatments on the same day post filleting on the photo data as it may not be able to pick up the detail witnessed by the naked eye, including translucency. This may be due to the loss in detail in the photographs when they are converted between *jpeg format that uses an RGB colour coding system to a *psd format that is associated with the photoshop™ program and uses an L*a*b* colour system to obtain the colour variables (Einen *et al.,* 1998).

4.3.2 Lipid Oxidation (TBARS)

Lipid oxidation in the fillets was quantified by the concentration of TBARS indicator compound malonaldehyde (MDA). The fillets from the fish fed the 30% *spirulina* diets had the highest oxidation levels of fatty acids. This could be due to the low feed intake of the diet when compared to the control and the 20% *spirulina* diets, which resulted in a low intake of antioxidants and vitamin E, which could have had a benefit of reducing the lipid oxidation in the diet over the storage period. Studies by Hamre *et al.,* (2003) have

demonstrated the benefit of high vitamin E diets that reduce lipid oxidation levels in fillets or Norweigian spring spawning herring (*Clupea harengus* L.). Several other studies have confirmed this observation, indicating that increasing vitamin E levels in feed results in less lipid oxidation of unsaturated fatty acids, and leads to a reduction in the production of "off" odours and flavours often associated with fillets that have undergone long term storage (Chaiyapechara, 2003). The fish fed the 20% *spirulina* diet had comparable TBARS levels after the eight day storage period and there was no significant difference from the control observed. Based on the feed data, there is evidence that the fish fed the 20% *Spirulina* diet consumed less vitamin E than the fish fed the control diet, this may give suggestions that the antioxidants present in the microalgae may have gone some way in protecting the polyunsaturated fatty acids in the fillets from lipid oxidation, although this has not been quantified further investigations could be carried out to determine the levels of vitamin E in the fillets of the fish fed the different dietary formulations and this will provide information as to how far the microalgae antioxidants go in providing protection against lipid oxidation in the fillets.

5 Conclusion

The commercial production of microalgae in South Australia is still in its infancy, and it has not reached a stage where there are products ready to be purchased by the consumer. Observations have also shown that total biomass production is very low, and will not be able to meet the demand that would be generated by the aquaculture industry. The price of microalgae is also still very high with the cheapest microalgae available in Australia at A$42kg^{-1} compared to A$2kg^{-1} for fish meal (index mundi, 2010). Optimism can be derived from knowing that economies of scale that can be achieved from increased production of microalgae and larger product focus will be possible in the near future. There is a greater need for more studies on microalgae application in aquaculture and it is possible that microalgae can be used as an alternative protein source resulting from microalgal biorefinery process for aquafeeds.

The use of *spirulina* in the feed trial has provided evidence that taste is a contributing factor towards the acceptability of microalgae formulated feeds by *S. lalandi*. The relatively low growth rates observed in the feed trial were attributable to the low water temperature conditions the fish were cultured in. Despite the low consumption rates of the fish in during the feed trial, the performance of the diet formulations were comparable to each other, firstly the growth rates were not significantly different from each other, secondly fish condition was not significantly different between the fish given the different dietary treatments, and thirdly fillet quality and storage was not significantly affected by the use of microalgae as an ingredient in the fish feed. The combination of a new feed formulation posed challenges for the experiment and this delayed the acceptability of the diets by the fish due to the low intestinal and gut activity that has generally been observed in the fish

during low temperature conditions. The performance of the 20% *spirulina* diet have indicated that improvements could be made in the experimental design of studies that observe the use of microalgae in feed trials particularly for fish that primarily prey on smaller schooling fish as they would have more defined taste preferences. The high activity of *S. lalandi* make it a fish that has high energy demands and it is imperative that feed trials that are conducted with the aim if studying the growth rates of the fish be carried out in conditions that encourage high feeding rates.

Future directions for this study would be to use of younger fish, preferably fingerlings to examine the acceptability of microalgae derived diets. This is because the sub-adult organisms may have been used to the taste of the control diet and hence their easy adaptability towards it during winter temperatures; it also allows for tracking of even the smallest changes in growth patterns and physiology. The feed trials would be carried out over a longer period of time to examine the long term effect of the microalgae consumption on the fish. Whole body proximate analysis on the feed and on the fish would be carried out to determine the effect of the microalgae and the fish.

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7 Appendix

7.1 Microalgae biomass analysis Results from the National Measurement Institute

Australian Government

National Measurement Institute

REPORT OF ANALYSIS

Report No. RN803540

Client	: FLINDERS UNIVERSITY	**Job No.**	: FLIN11/100618
	DEPARTMENT OF MEDICAL BIOTECHNOLOGY	**Quote No.**	: QT-01453
	NEW HEALTH SCIENCES BUILDING	**Order No.**	:
	LEVEL 4	**Date Sampled**	: 15-JUN-2010
	REGISTRY ROAD	**Date Received**	: 18-JUN-2010
Attention	: Kopano Machailo	**Sampled By**	: CLIENT
Project Name	:		
Your Client Services Manager	: Tim Stobaus	**Phone**	: (03) 9644 4849

Lab Reg No.	Sample Ref	Sample Description
V10/017028	Sample 1	Isochrysis-Nannochloropsis Mixture 300g 15/6
V10/017029	Sample 2	Spirulina Sp. Biomass 300g 15/6

Lab Reg No.		V10/017028	V10/017029			
Sample Reference	Units	Sample 1	Sample 2			Method
Proximates						
Fructose	g/100g	< 0.2	< 0.2			VL295
Glucose	g/100g	< 0.2	< 0.2			VL295
Sucrose	g/100g	0.4	< 0.2			VL295
Maltose	g/100g	< 0.2	< 0.2			VL295
Lactose	g/100g	< 0.2	< 0.2			VL295
Total Sugars	g/100g	< 1	< 1			VL295
Moisture	g/100g	6.6	5.3			VL298
Fat (Mojonnier extraction)	g/100g	8.4	4.6			VL302
Protein (N x 6.25)	g/100g	9.5	67.1			VL299
Ash	g/100g	56.5	6.2			VL286
Carbohydrates	g/100g	19	17			
Mono trans fats	g/100g	< 0.1	< 0.1			VL289
Mono-unsaturated fat	g/100g	5.0	0.5			VL289
Omega 3 fats	g/100g	0.8	< 0.1			VL289
Omega 6 fats	g/100g	0.6	2.2			VL289
Poly trans fats	g/100g	< 0.1	< 0.1			VL289
Poly-unsaturated fat	g/100g	1.3	2.2			VL289
Trans fats	g/100g	< 0.1	< 0.1			VL289
Saturated Fatty Acids						
C4:0 Butyric	%	< 0.1	< 0.1			VL289
C6:0 Caproic	%	< 0.1	< 0.1			VL289
C8:0 Caprylic	%	< 0.1	< 0.1			VL289
C10:0 Capric	%	< 0.1	< 0.1			VL289
C12:0 Lauric	%	< 0.1	< 0.1			VL289
C14:0 Myristic	%	2.5	0.1			VL289
C15:0 Pentadecanoic	%	0.2	< 0.1			VL289
C16:0 Palmitic	%	16.7	35.8			VL289
C17:0 Margaric	%	0.1	0.2			VL289
C18:0 Stearic	%	1.0	1.4			VL289
C20:0 Arachidic	%	0.1	< 0.1			VL289

REPORT OF ANALYSIS

Lab Reg No.		V10/017028	V10/017029			
Sample Reference	Units	Sample 1	Sample 2			Method
Saturated Fatty Acids						
C22:0 Behenic	%	0.1	< 0.1			VL289
C24:0 Lignoceric	%	0.2	< 0.1			VL289
Total Saturated	%	21.0	37.9			VL289
Mono-unsaturated Fatty Acids						
C14:1 Myristoleic	%	< 0.1	< 0.1			VL289
C16:1 Palmitoleic	%	2.7	9.0			VL289
C17:1 Heptadecenoic	%	< 0.1	< 0.1			VL289
C18:1 Oleic	%	57.1	2.6			VL289
C20:1 Eicosenic	%	1.0	< 0.1			VL289
C22:1 Docosenoic	%	0.5	< 0.1			VL289
C24:1 Nervonic	%	0.7	0.1			VL289
Total Mono-unsaturated	%	62.0	11.8			VL289
Poly-unsaturated Fatty Acids						
C18:2w6 Linoleic	%	6.3	14.4			VL289
C18:3w6 gamma-Linolenic	%	< 0.1	34.2			VL289
C18:3w3 alpha-Linolenic	%	8.5	0.2			VL289
C20:2w6 Eicosadienoic	%	0.2	0.3			VL289
C20:3w6 Eicosatrienoic	%	< 0.1	0.5			VL289
C20:3w3 Eicosatrienoic	%	< 0.1	< 0.1			VL289
C20:4w6 Arachidonic	%	0.1	< 0.1			VL289
C20:5w3 Eicosapentaenoic	%	0.4	< 0.1			VL289
C22:2w6 Docosadienoic	%	< 0.1	< 0.1			VL289
Omega 3 Fatty Acids	%	9.8	0.3			VL289
Omega 6 Fatty Acids	%	6.9	49.7			VL289
C22:4w6 Docosatetraenoic	%	< 0.1	0.3			VL289
C22:5w3 Docosapentaenoic	%	< 0.1	< 0.1			VL289
C22:6w3 Docosahexaenoic	%	0.9	< 0.1			VL289
Total Poly-unsaturated	%	16.7	50.0			VL289
Total Mono Trans Fatty Acids	%	0.1	< 0.1			VL289
Total Poly Trans Fatty Acids	%	0.1	0.2			VL289
P:M:S Ratio		0.8:2.9:1	1.3:0.3:1			VL289

Dr. Nahar Syeda, Analyst
Food Composition - Vic

Neil Menz, Analyst
Food Composition - Vic

Paul Adorno, Section Manager
Food Composition - Vic

REPORT OF ANALYSIS

Dianne Laugher, Analyst
Organics - Vic

8-JUL-2010

Lab Reg No.		V10/017028	V10/017029			
Sample Reference	Units	Sample 1	Sample 2			Method
Outsourced Tests						
Alanine	mg/g	4.49	37.55			
Arginine	mg/g	3.52	39.14			
Aspartic Acid	mg/g	6.97	49.73			
Glutamic Acid	mg/g	8.52	76.05			
Glycine	mg/g	3.65	23.50			
Histidine	mg/g	1.01	8.47			
Isoleucine	mg/g	3.02	30.40			
Leucine	mg/g	5.47	47.10			
Lysine	mg/g	3.16	25.28			
Methionine	mg/g	0.46	11.08			
Phneylalanine	mg/g	3.45	24.72			
Proline	mg/g	4.26	20.07			
Serine	mg/g	3.09	24.86			
Threonine	mg/g	3.28	25.78			
Total Amino Acids	mg/g	59.96	498.32			
Tyrosine	mg/g	1.86	20.91			
Valine	mg/g	3.75	33.69			

V10/017028
-V10/017029
Amino acids determined by Australian Proteome Analysis Facility (APAF), Macquarie University Sydney.
Values refer to Amino Acid residue mass in protein (molecular weight minus water)
See attached APAF Report (30 June 2010)

Tim Reddan
Laboratory Services Unit - Vic

7.2 Biomss gain per fish

Week 0

ANOVA[a]

fish weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19267.302	2	9633.651	.597	.552
Within Groups	1695059	105	16143.416		
Total	1714326	107			

a. week sampled = week 0

fish weight[b]

Student-Newman-Keuls [a]

diet fed	N	Subset for alpha = .05
		1
20% spirulina	36	733.7694
control	36	742.1361
30% spirulina	36	765.3444
Sig.		.544

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 36.000.

b. week sampled = week 0

Week 8

ANOVA[a]

fish weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	85626.705	2	42813.352	3.167	.047
Within Groups	1270750	94	13518.619		
Total	1356377	96			

a. week sampled = week 8

fish weight[c]

Student-Newman-Keuls [a,b]

diet fed	N	Subset for alpha = .05
		1
30% spirulina	32	683.0544
20% spirulina	34	684.5071
control	31	747.5042
Sig.		.072

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 32.286.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. week sampled = week 8

Fillet Yield

ANOVA

fillet yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	42249.644	2	21124.822	7.172	.002
Within Groups	123710.933	42	2945.498		
Total	165960.578	44			

fillet yied

Student-Newman-Keuls [a]

diet	N	Subset for alpha = .05	
		1	2
20% spirulina	15	298.6000	
30% spirulina	15	303.4000	
control	15		365.8667
Sig.		.810	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

7.3 VSI and HSI

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
VSI (%)	Between Groups	2.105	2	1.052	3.967	.026
	Within Groups	11.142	42	.265		
	Total	13.246	44			
HSI (%)	Between Groups	.596	2	.298	5.304	.009
	Within Groups	2.358	42	.056		
	Total	2.954	44			

VSI (%)

Student-Newman-Keuls [a]

		Subset for alpha = .05	
diet	N	1	2
30% spirulina	15	4.0988	
20% spirulina	15		4.4808
control	15		4.6077
Sig.		1.000	.504

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

HSI (%)

Student-Newman-Keuls [a]

		Subset for alpha = .05	
diet	N	1	2
30% spirulina	15	.5619	
control	15		.8035
20% spirulina	15		.8083
Sig.		1.000	.956

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

7.4 Condition Index

ANOVA

fish weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41407.887	2	20703.943	1.339	.264
Within Groups	3123443	202	15462.588		
Total	3164851	204			

fish weight

Student-Newman-Keuls [a,b]

diet fed	N	Subset for alpha = .05
		1
20% spirulina	70	709.8420
30% spirulina	68	726.6197
control	67	744.6199
Sig.		.234

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 68.311.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

7.5 Total apparent feed intake

ANOVA

total apparent feed intake

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	674008.5	2	337004.262	5.991	.037
Within Groups	337530.3	6	56255.044		
Total	1011539	8			

total apparent feed intake

Student-Newman-Keuls [a]

diet	N	Subset for alpha = .05	
		1	2
30% spirulina	3	816.4933	
20% spirulina	3	1099.2333	1099.2333
control	3		1484.2167
Sig.		.195	.094

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

7.6 Tank biomass

Week 0

ANOVA[a]

tank biomass

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	231207.6	2	115603.810	2.965	.127
Within Groups	233939.2	6	38989.860		
Total	465146.8	8			

a. sampling week = week 0

tank biomass[b]

Student-Newman-Keuls [a]

diet fed	N	Subset for alpha = .05
		1
20% spirulina	3	8805.2333
control	3	8905.6333
30% spirulina	3	9184.1333
Sig.		.124

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

b. sampling week = week 0

ANOVA[a]

tank biomass

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	615649.3	2	307824.641	.611	.574
Within Groups	3024341	6	504056.822		
Total	3639990	8			

a. sampling week = week 8

tank biomass[b]

Student-Newman-Keuls [a]

diet fed	N	Subset for alpha = .05
		1
20% spirulina	3	8083.8667
control	3	8536.5667
30% spirulina	3	8702.8000
Sig.		.566

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

b. sampling week = week 8

7.7 Colour analysis

7.7.1 L* day 0

ANOVA[a]

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.378	2	.689	.147	.863
Within Groups	196.267	42	4.673		
Total	197.644	44			

a. colour parameter = L, day stored = day 0

colour parameter[b]

Student-Newman-Keuls [a]

diet fed	N	Subset for alpha = .05 1
30% spirulina	15	56.9333
20% spirulina	15	57.0000
control	15	57.3333
Sig.		.868

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

b. colour parameter = L, day stored = day 0

L* day 8

ANOVA[a]

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.711	2	3.356	.586	.561
Within Groups	240.533	42	5.727		
Total	247.244	44			

a. colour parameter = L, day stored = day 8

colour parameter[b]

Student-Newman-Keuls [a]

diet fed	N	Subset for alpha = .05 1
control	15	63.0000
30% spirulina	15	63.6000
20% spirulina	15	63.9333
Sig.		.539

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 15.000.

b. colour parameter = L, day stored = day 8

7.7.2 a* day 0

ANOVA[a]

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.044	2	8.022	1.485	.238
Within Groups	226.933	42	5.403		
Total	242.978	44			

a. colour parameter = a, day stored = day 0

colour parameter(b)

Student-Newman-Keuls

	N	Subset for alpha = .05
diet fed	1	1
20% *spirulina*	15	11.4000
30% *spirulina*	15	11.7333
control	15	12.8000
Sig.		.237

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 15.000.
b colour parameter = a, day stored = day 0

a* day 8

ANOVA(a)

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22.711	2	11.356	6.232	.004
Within Groups	76.533	42	1.822		
Total	99.244	44			

a colour parameter = a, day stored = day 8

ANOVA(a)

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.178	2	.089	.028	.972
Within Groups	132.267	42	3.149		
Total	132.444	44			

a colour parameter = b, day stored = day 0

7.7.3 b* day 0

ANOVA(a)

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.178	2	.089	.028	.972
Within Groups	132.267	42	3.149		
Total	132.444	44			

a colour parameter = b, day stored = day 0

colour parameter(b)

Student-Newman-Keuls

	N	Subset for alpha = .05
diet fed	1	1
30% *spirulina*	15	10.8000
control	15	10.9333
20% *spirulina*	15	10.9333
Sig.		.977

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 15.000.
b colour parameter = b, day stored = day 0

b* day 8

ANOVA(a)

colour parameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.533	2	20.267	6.828	.003
Within Groups	124.667	42	2.968		
Total	165.200	44			

a colour parameter = b, day stored = day 8

colour parameter(b)

Student-Newman-Keuls

	N	Subset for alpha = .05	
diet fed	1	2	1
30% *spirulina*	15	16.8667	
control	15		18.7333
20% *spirulina*	15		19.0000
Sig.		1.000	.674

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 15.000.
b colour parameter = b, day stored = day 8

7.8 TBARS

Day 0

ANOVA(a)

concentration MDA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	2	.000	.027	.973
Within Groups	.435	42	.010		
Total	.435	44			

a storage days = 0 days

concentration MDA(b)

Student-Newman-Keuls

diet fed	N	Subset for alpha = .05
		1
20% *Spirulina*	15	.2328
control	15	.2370
30% *spirulina*	15	.2415
Sig.		.970

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 15.000.
b storage days = 0 days

Day 8

ANOVA(a)

concentration MDA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.079	2	.040	2.600	.086
Within Groups	.639	42	.015		
Total	.718	44			

a storage days = 8 days

concentration MDA(b)

Student-Newman-Keuls

diet fed	N	Subset for alpha = .05
		1
control	15	.2338
20% *Spirulina*	15	.2502
30% *spirulina*	15	.3298
Sig.		.096

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 15.000.
b storage days = 8 days