Evaluating Spirulina as a protein source in Nile Tilapia (*Oreochromis niloticus*) grow-out diets

by

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A thesis submitted to the Department of Biology
In conformity with the requirements for the degree of Master of Science

Queen's University Kingston, Ontario, Canada
(May, 2016)

Abstract:

Aquaculture growth has intensified the need for a diversification of nutritionally appropriate aquafeed ingredients. The purpose of this study was to evaluate Spirulina, a blue-green microalgae, and soybean meal as the sole protein sources in grow-out Tilapia diets. We constructed 3 experimental diets with soybean meal and 0,15, 30, and 45% Spirulina (SBM, SP15, SP30, and SP45 respectively) as their main protein sources. We compared these diets to a commercial Tilapia diet (CC). Additionally, to evaluate the benefit of fishmeal inclusion, fishmeal was added (2 and 10%) to the most successful Spirulina containing diet (FM2, FM10). We evaluated these experimental diets based on their physical properties, palatability, growth potential, waste production, and overall cost. No significant differences in growth performance were found between any of the diets. Total ammonia nitrogen (TAN) and total phosphorus (TP) levels in each tank were significantly affected by diet ($p<0.05$). CC had significantly higher TP than the experimental diets and SP15 had significantly higher TAN than the other diets. Only CC was found to be significantly more palatable than the experimental diets, and Spirulina inclusion was inversely correlated to pellet stability. Lastly, SP15 was the most profitable experimental diet. We recommend eliminating fishmeal from grow-out Tilapia diets in favour of soybean meal and Spirulina. Spirulina should, however, be limited to 15% to avoid the negative effects it has on stability and profitability, and its possible effect on feed intake.

Acknowledgments:

I'd like to start by thanking my supervisor, Dr. Bruce Tufts, for giving me the opportunity to pursue my research interests. I appreciate the freedom you allowed me and the resources you provided.

Thank you to my two committee members: Dr. Daniel Lefebvre and Dr. Yuxiang Wang- your support was and continues to be much appreciated.

To my lab mates, past and present, thank you for putting up with me and thank you for all of the laughs. Without all of you, my time here would not have been as fun or as productive.

Lastly, I'd like to extend a massive thank you to my family and friends. I threatened to quit many times and without all of your efforts, this document would not exist. Thank you for you kind (and not so kind) words of encouragement.

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List of Abbreviations

AER- Absorption efficiency ratio

CA- Citric acid enhanced feed

CAN$- Canadian dollar

CC- Commercial control

DB- Dried basil enhanced feed

DO- Dissolved oxygen

EAA- Essential amino acids

FAO- Food and agriculture organization

FCR- Feed conversion ratio

FM2- Fishmeal enhanced diet 2%

FM10- Fishmeal enhanced diet 10%

GDP- Gross domestic product

RAR- Relative absorption rate

SBM- Soybean meal control diet

SGR- Specific growth rate

SP15- 15% Spirulina experimental feed

SP30- 30% Spirulina experimental feed

SP45- 45% Spirulina experimental feed

TAN- Total ammonia nitrogen

TP- Total phosphorous

Chapter 1. Introduction

1.1. Global overview of aquaculture growth

Aquaculture is one of the fastest growing food production sectors in the world and is expected to continue growing for decades to come. Since the 1960s, aquaculture output has increased by 8.2% per year, surpassing both capture fisheries (1.3% per year) and agricultural meat production (2.6% per year) (FAO, 2013). Aquaculture production has also outpaced global population growth, allowing for a 7.1% per year rise in per capita aquaculture (product) consumption, equivalent to a 3.2% increase in global fish supply (FAO, 2014). As the global population and GDP increase, demand for fisheries product is unlikely to slow. The Food and Agriculture Organization (FAO) therefore estimates that fish farms will have to increase output by another 50 million tonnes in the next 15 years to satisfy demand (FAO, 2013). In order to sustainably reach this target, barriers to aquaculture growth and inefficient uses of aquaculture resources must be addressed.

1.2. Barriers to sustainable aquaculture

Any method of food production should be measured against its environmental impact and its use of natural resources. Similar to agriculture, the main issues with aquaculture stem from its reliance on inputs, and its harmful outputs. Aquaculture largely takes place within existing water bodies, and relies on the natural ecosystem's sustained health to provide ideal growth conditions such as temperature, pH, dissolved oxygen content etc. The species cultured through aquaculture systems can, however, put the ecosystem at risk through the introduction of exotic species, pathogens, antibiotics, nutrient loading, and general habitat destruction which can affect growth conditions such as pH and dissolved oxygen (Mente et al. 2006). Open water aquaculture is thus inextricably tied to the health of its surrounding environment.

Recently, there has been a trend towards land based, recirculating aquaculture systems. This protects natural populations from direct interference from aquaculture practices but fails to address aquaculture's biggest sustainability barriers. Regardless of culture location, intensive aquaculture requires the input of fish feed (aquafeed), which is a resource intensive product. Furthermore, the conversion of fish feed to edible matter will result in organic and inorganic waste products, which requires collection and treatment. These related issues are two of the most important, and potentially constraining factors facing inland aquaculture growth.

Due to a stagnation of capture fisheries, any increase in seafood demand will likely result in increased aquaculture demand, leading to an increase of aquaculture inputs and waste outputs. By 2030, aquaculture production is likely to provide approximately 62% of all food fish, and unless widespread changes to practice are adopted, the impact on the environment, direct and indirect, will increase (Naylor et al. 2000).

1.3. The role of feed in sustainable aquaculture

The majority of aquaculture relies on feed inputs in the form of aquafeed. Approximately 70% of all aquaculture production are direct-fed species, which is roughly equivalent to 35.7 million tonnes of product. Of these direct-fed species, 68% are dependent on commercially produced feed products and consumed an estimated 39.6 million tonnes of aquafeed in 2012 (Tacon and Metian, 2015). The aquaculture farms relying on commercial feeds are intensive production facilities. Whereas European and North American aquaculture is virtually entirely intensive and exclusively fed using commercial aquafeeds, Asian aquaculture is undergoing an intensification transition (FAO, 2009). This has allowed the aquafeed industry to grow at an average rate of 10.3% per year since 2000. By 2025, aquafeed production is expected to reach 87.1 million tonnes (Tacon and Metian, 2015).

The predicted rise in aquafeed production will not be without environmental consequences. Aquafeed is formulated from natural ingredients requiring inputs, such as fertilizer and water, for growth and harvesting. Aquafeeds are also ultimately the source of aquaculture waste. High functioning aquafeeds packed with protein and nutrients, are not fully utilized by the aquacultured species, resulting in the release of nutrient rich waste to the environment or recirculating system. Aquafeed therefore represents one of the most environmentally troublesome portions of the aquaculture process, and is a major barrier to aquaculture sustainability.

Aquaculture must reconsider how to feed fish, while maximizing profits, and minimizing waste and environmental impacts. Traditional protein ingredients, such as fishmeal and soybean meal, are competitive resources, and their costs or availability may impede aquaculture growth. Additionally, relying on any ingredient too heavily leads to risk associated with supply, price and quality fluctuations (Glencross et al. 2007). The FAO has therefore stated that research into environmentally and socially responsible, high quality, and cost effective feedstuffs should be pursued (FAO, 2012).

1.4. Fishmeal

Fishmeal is the major protein component in traditional aquafeeds. Fishmeal is produced by cooking, pressing, drying, and milling whole fish and fisheries by-product. It has an appropriate balance of essential amino acids, is palatable and digestible by a wide array of fishes, and has been relatively accessible for a number of decades.

Unfortunately, the way fishmeal is harvested is associated with both ecological and economic issues, which have been aggravated by the increased pressure of a growing aquaculture industry.

Since fishmeal is harvested from wild populations, both production limits and consequences for when those limits are surpassed exist. The species targeted for fishmeal production are low value pelagic forage fish (ex. Clopeiformes and Scombroidei) not otherwise destined for human consumption or harvested in excess of food fish demand (Bose et al. 1991). Many fishmeal fisheries stocks, such as the anchoveta (*Engraulis ringens*) stocks in the Southeast Pacific and the herring stocks in the North Atlantic, are considered fully exploited (FAO, 2007). In removing a large number of individuals from a specific trophic level, their ability to perform ecological functions is limited. Specifically, since the fish harvested for fishmeal have an average trophic level between 2.8-3.0, they occupy the roles of both predator and prey species (Tacon and Metian, 2009). In years where natural or fishery induced mortality rates fluctuate, the availability of these targeted species affects the biomass and fitness of higher and lower trophic levels (Naylor et al. 2001). The harvesting of pelagic forage fish for fishmeal production therefore risks the health of targeted populations and their ecosystems. Government-imposed quotas based on maximum sustainable yield calculations are intended to limit the risk of negative environmental impacts and promote sustainable use of fishmeal fisheries. Despite the quota system, the FAO suggests that our reliance on fishmeal for aquaculture is undermining both marine biodiversity and human food security (FAO, 2012).

Fishmeal is also subject to price fluctuations related to the manner in which it is produced and the extent to which aquaculture relies on it (Goncalves Leal et al. 2010). Given the lack of equally effective and available alternatives, fishmeal is considered to be an economically inelastic product, meaning that in the face of moderate price increases, demand will not decrease, and prices will not have a compensatory fall (Rumsey, 2003). This has allowed fishmeal to undergo sustained long-term price increases, punctuated by short-term supply related price spikes (Appendix A1). In the South Pacific, El Niño events cause price spikes, as oceanic water temperature fluctuations affect Peruvian and Chilean anchoveta reproduction and recruitment rates, limiting fisheries output (Sheperd and Jackson, 2013). In 2006, fishmeal prices in the United States more than doubled during an El Niño year as competition for the reduced fishmeal supply increased (Tacon and Metian, 2009). Between 2010 and 2030, the real price of fishmeal is expected to rise by 90% (World Bank, 2013). Given that aquafeed is typically the largest variable cost in an aquaculture facility, fishmeal prices risk limiting aquaculture growth (Ajiboye and Yakubu, 2010; Naylor et al. 2009).

Expanding aquaculture demands for aquafeeds is expected to push the prices for this limited fishmeal supply upwards. Unless a suitable alternative to fishmeal is found, growth in the aquaculture sector is likely to push the price for a fixed amount of fishmeal too high, and encourage overfishing in poorly regulated areas (Naylor et al. 2009).

1.5 Common protein alternatives

Despite being a desirable ingredient for aquafeed, fishmeal is not essential. Rather, it is a nutritionally balanced and cost effective protein source that has become progressively more expensive and increasingly scarce. Therefore, fishmeal has gradually been replaced with other competitive ingredients. Several effective alternatives to fishmeal have been identified, but all are subject to limitations that restrict their use in fish diets. These alternatives have been both plant and animal based, including terrestrial animal by-products (chicken offal, blood-meal etc.), and various agricultural by-products (soybean meal, corn gluten meal etc.). The use of these products, and a more judicious and strategic use of fishmeal products, has resulted in a decrease in global fishmeal inclusion levels. Sheperd and Jackson (2013) suggest that fishmeal's price increases will continue to push certain users out of the market. Therefore, eliminating fishmeal from omnivorous species aquafeed, and limiting competition for the resource, is of great importance.

1.5.1. Soybean meal

Soybean protein is the most important plant based fishmeal alternative. Soybean meal is one of the products made from a cake of high protein quality leftover after the extraction of soybean oil from a soybean. Soybean cake can also be used to create products such as soybean flour, soy protein concentrate, and soy protein isolate, all of which have been studied in fish diets (Gatlin et al. 2007). Wantanabe (2002) suggests that given soybean meal's nutritional effectiveness, availability, and cost effectiveness, many aquaculture nutritionists should aim to completely remove fishmeal in favour of soybean meal. Soy protein inclusion rates in aquafeed have increased, but diets with soy as their only source of protein remain uncommon, even in omnivorous species.

Completely soy-based protein diets are unusual given conflicting evidence regarding its use as the main protein source in omnivorous and carnivorous fish aquafeeds (Ogello et al. 2014). El-Sayed (1999) suggests that soybean meal can replace between two thirds and 100% of fishmeal in aquafeeds depending on species, dietary protein levels, source, processing methods, and the culture systems used. In carnivores, over 50% substitution success is rare. Colburn et al. (2012) found that a 50% substitution of fishmeal with soybean meal and soy protein concentrate is possible without significant reductions in growth and fish condition, but 100% substitution in Atlantic Cod (*Gadus morhua*) diets is not recommended. The evidence for omnivorous species, such as Tilapia, is more promising, with confirmation that soy based diets with added methionine does not negatively affect growth (Zhao et al. 2010). Implementing soy-based diets therefore appears to be a more achievable goal for omnivorous species than carnivorous ones.

Although soybean meal may not be a perfect replacement for fishmeal in all finfish diets, it is more economically stable. Compared to fishmeal, soybean meal is less expensive, and displays less short and long term price fluctuations (Appendix A1). Between December 2013 and December 2014, the price of fishmeal fluctuated between 1656.80 CAN$ and 2751.32 CAN$ while soybean meal varied between 526.69$ and 482.59$ (Index mundi, accessed October 2015). Additionally, unlike fishmeal, soybean meal is an agricultural crop, that's supply can be somewhat managed by demand.

Soybean meal does, however, have economic and ecological drawbacks. Firstly, soybean meal is a competitive crop that is heavily used in terrestrial and aquatic animal feeds. This is concerning considering that the demand for agricultural aquafeed ingredients like soybean meal is increasing faster than the rate of supply has increased (FAO, 2009). Therefore, similar to fishmeal, an increase in soybean meal demand over supply will lead to a price increase that will likely affect developing countries and small scale producers (FAO, 2009). A rise in demand for soybean meal will also likely push producers to undertake biologically unsound, or risky production methods, resulting in aquaculture acting as a driving factor in unsustainable agriculture practices to increase yield, as it has with fishmeal (Foley et al. 2011). Lastly, an overreliance on a terrestrial crop like soybean meal could leave the aquaculture industry at the mercy of the long-term effects of climate change on agricultural yields. Already, the FAO suggests that climate change has resulted in lower crop yields for a number of important agricultural crops used in aquaculture diets (FAO, 2009). Considering all of the above issues, the FAO does not expect soybean meal prices to return to levels similar or below pre-2000 (FAO, 2009). A general diversification of the types of feed ingredients used in aquaculture should be pursued.

1.6 Qualities of a good alternative

A good alternative protein product satisfies the most important goal of sustainable aquaculture: to maximize the conversion of feed to edible fish (Bostock et al. 2010). The effectiveness of a feed is affected by the fish's sensory ability to first locate and identify the feed; its physical ability to capture, handle, and ingest the feed; and its biochemical capacity to digest and utilize the feed's ingredients. Novel feed ingredients are often tested against traditional ingredients through a series of experiments that evaluate feed ingredient appropriateness using the above-mentioned parameters (Glencross et al. 2007). Once determined to be biologically suitable, the ingredient must also be assessed according to its functionality, availability, and cost.

1.6.1. Palatabilty

Palatability is the measure of whether an organism will accept or reject a feed based on taste. A palatable feed will increase feed intake, lessen feed waste, and increase growth (Toften et al. 1995). Given the questionable nutritional effectiveness of many alternative protein products when compared to fishmeal, sustaining feed and nutrient intake through palatability is necessary (Refstie et al. 1997).

Feeding is an elective process. Although a fish detects food visually and via olfaction and chemical cues, taste and texture are the determinants of whether or not food will be accepted and swallowed (Adron and Mackie, 1978). Experiments designed to measure fish taste preference can be split into two groups: long-term growth experiments evaluating feed intake, and feed preference experiments (Dias et al. 1997; Adron et al. 1973). When presented with feed choices, fish are capable of preferentially ingesting the feed they consider to be the most palatable (Toften et al. 1995; Gaber, 2005; Pereira-da-Silva and Pezzato, 2000). Fish are also able to activate self feeders, and have demonstrated higher feeding rate on one feeder over another when there is a preference for one feed over another (Montya et al. 2011, Boujard and Le Gouvello 1997). Feed preference, and ultimately relative palatability, can therefore be tested through single feed or dueling feed scenarios.

Incorporating feed enhancers is known to increase the palatability of otherwise unpalatable ingredients (Madalla, 2008). Compounds such as organic acids, which are commonly found in aquatic organism extracts, are recognized feed enhancers for many fish species (Hidaka et al. 1992). Citric acid, in particular, has been found to be helpful in aquafeed storage and especially palatable to Tilapiines, capable of overriding their natural taste preferences (Kumar et al. 1997; Adams et al. 1988, Xie et al. 2003; Kasumyan and Prokopova, 2001). Tilapia species presented with unpalatable plant based feeds enhanced with 10^{-2} to 10^{-6}M of citric acid were found to display increased feed intake, and preferentially selected the enhanced plant based feed over an animal based feed 84% of the time (Xie et al. 2003; Adams et al. 1988).

Less well-studied feed enhancers include herbs and spices. Incorporating herbs, such as basil, increased the palatability of feeds containing fishmeal, meat meal, and soybean meal resulting in increased feed intake, decreased feed waste, and increased growth (Dakar et al. 2008). Additionally, herbs can contribute towards the odour and flavour of the fish through the presence of volatile and fixed oils, and may present the fish with added health benefits (Ji et al. 2007, Acar et al. 2015). Therefore, in the interest of promoting potentially unpalatable alternative protein sources, well-established feed enhancers can be employed, and novel feed-enhancers with potential added benefits should be explored.

1.6.2. Growth and digestibility

In aquaculture, growth is ultimately the feed performance measure of the most interest. An increased yield or decreased time to harvest, can justify more expensive feed ingredients, or more expensive or laborious production methods. Growth will be affected by both the feed's digestibility and nutritional profile.

Digestibility refers to the ability to absorb and assimilate ingested feed. Poor assimilation will lead to reduced or no fish growth, even if the feed is nutritionally adequate. Undigested feed material is not utilized for growth and is excreted. Digestibility therefore impacts the appropriateness of a feed ingredient, diet formulation, and waste production predictions (Koprucu and Ozdemir, 2005; Amirkolaie, 2005).

Whereas fishmeal is considered digestible, poor digestibility has been described for a number of plant ingredients (Gomes et al. 1995). Crude fibre, which serves as structural support for many plants, anti-nutritional factors, which are inherent to many plant species, and high carbohydrate content, can all lead to poor digestibility in fish (Francis et al. 2001). As many of the proposed alternatives to fishmeal are plant species, limiting these poor-digestibility contributing factors is crucial. Digestibility can also be affected by a number of other factors including species, fish size or age, sampling methods, time to adjust to the new diet ingredient, and added binding agents (Sarker et al. 2016). Failing to optimize all of these factors can lead to the waste of ingested diet ingredients, and therefore sub-optimal conversion of feed to edible fish flesh.

A digestible diet that does not contain the proper balance of amino acids is also ineffective. Proteins are the major organic material in fish tissue, and of the utmost interest in aquafeed development. Fish require ten amino acids to be delivered through the protein in their diet: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (De Silva and Anderson, 1998). When present in the correct relative proportion, efficient protein synthesis can take place.

1.6.3. Waste

Feed material that is not used by the fish is excreted as waste. These wastes are delivered to the ecosystem in the form of uneaten fish feed and fish feces, and are nitrogen and phosphorous intensive. Cultured salmonids lose approximately 28% of the nitrogen and 50% of the phosphorous provided to them in their food in a dissolved form (Mente et al. 2006). When factoring in feed lost as feces, cultured fish can lose an additional 50% of ingested nitrogen in the form of dissolved inorganic nitrogen (Yokoyama and Ishihi. 2010). Nutrient enrichment delivered to a coastal ecosystem in these forms can lead to an increase in primary productivity and increased risk of eutrophication. The damage is usually limited to a 20-50m radius around coastal fish pens, but reports of the waste travelling up to 100m are common in high current velocity areas (Mente et al. 2006). The accumulation of the wastes leads to changes in the sediment, resulting in a lowered redox potential, high organic content, and a buildup of nitrogenous and phosphorous compounds (Mente et al. 2006). Limiting nutrient enrichment is therefore important in terms of both ecosystem conservation and the maintenance of water quality for the aquaculture industry.

Since aquaculture waste ultimately originates from aquafeeds, it is appropriate to look to aquaculture diets for waste reduction and mitigating measures. Researchers suggest that this be done through improving diet formulation (Cho and Bureau, 2001). Nitrogenous waste is produced through the transformation of nitrogen in protein to ammonia. Amino acids of ingested proteins that are not used in body synthesis are deaminated. The resulting nitrogen waste is excreted through the gills and urinary pore. When fish are fed a poor amino acid profile, or part of the amino acid profile is poorly digested, less protein synthesis can occur, and the unused amino acids are deaminated and excreted. Tailoring the amino acid profile of a feed for a particular species, and ensuring the digestibility of the feed ingredients, can lead to improved nitrogen retention, and decreased deamination and nitrogenous waste excretion.

Aquafeed is also the main source of dietary phosphorous for fish (Auer et al. 1986; Foy and Rosell, 1991). Excess dietary phosphorus is excreted in dissolved form through the gills or kidney, or through particulate matter in feces (Nakashima and Leggett, 1980; Jahan et al. 2003). Literature concerning the reduction of phosphorus waste is limited (Buyukates et al. 2000). What is clear, however, is that the use of plant products low in phosphorus content but relatively high in phosphorus availability over animal products such as fishmeal, may be useful in diet formulations intended to limit phosphorus waste (Buyukates et al. 2000). The phosphorus content of soybean meal, for example, is high, but its availability is limited by the antinutritional factor, phytate. Supplementing plant meals with phytase renders the soybean meal more digestible, and the phosphorus more bioavailable (Riche and Brown, 1996). Detrimental phytate levels can also be reduced through removing a cereal's outer endosperm, and with heat treatment (Francis et al. 2001). Phosphorus waste output can therefore be reduced through the careful selection of ingredients, the treatment of ingredients against antinutritional factors, and the optimization of digestible phosphorus content of the diet to meet requirements of the fish (Cho and Bureau, 2001).

1.6.4. Availability, accessibility, and functionality

A feed's ingredients must also be functional, consistently available, and cost effective. Commercial feed is formulated to provide a balanced diet, and to fulfill the physical and economic requirements of fish farmers and feed producers alike. The majority of commercial feeds are processed into dry pellets. Pelleted feeds result in less waste, and their physical properties (including density and water stability) can aide in identifying appropriate feeding rations. Feed stability can be imparted to a feed through its ingredients. Ingredients such as flour, other starches, and guar gum are known binders, increasing the feed's ability to maintain integrity in the water column. Conversely, some products are suspected to decrease water stability, or are dense enough to reduce a pellet's ability to float. Ingredients can therefore be selected or adopted sparingly according to their specific binding abilities.

Secondly, every input required to produce edible fish flesh must result in the maximization of value added. The challenge in adopting a novel feed ingredient in an aquafeed is that the product must either contribute to market demand of the end product or result in a larger profit potential. The use of an alternative protein product must result in some sort of competitive advantage (Milledge, 2011; Radmer and Parker 1994).

1.7 Tilapia

Tilapia is the common name given to a group of pure and hybrid species belonging to the family Cichlidae. Among this group, the most popular aquacultured species is the Nile Tilapia (*Oreochromis niloticus*) and its various hybrids. Currently, Tilapia is the most widely cultured species, and has experienced a 7-10% increase in production over the last 20 years (Fitzsimmons, 2010).

Tilapia are the second largest consumers of commercial aquafeed after carp (6.67 and 11.03 million tonnes respectively) and consume approximately 3% of the global supply of fishmeal (Tacon et al. 2006)(Tacon and Metian, 2015). Tacon and Metian (2015) estimate that as of 2000, 75% of Tilapia grown globally were fed commercial aquafeed and that by 2025, that percentage will have increased to 100%. Because Tilapia production is expected to double from 4.3 million tons to 7.3 million tons between 2010 and 2030 (World Bank2013 2030), it is necessary that we find an alternative to expensive, ecologically harmful, and unnecessary ingredients in Tilapia aquafeeds.

Tilapia owe their popularity to their hardiness, mild taste, and flexible diet. Tilapia are omnivorous and are able to convert increased proportions of carbohydrates in plant materials, withstand decreased protein quality, and conserve protein in their diets for growth (Stone, 2003). Tilapia do not require fishmeal as a part of their diet to maintain growth or health and are therefore a popular species for testing novel feed ingredients (Zhao et al. 2010). That being said, the unbalanced essential amino acid profiles and high levels of anti-nutritional factors that limit the inclusion of terrestrial plants in many finfish diets can also be problematic for Tilapia (Shiau et al. 1990; Maina et al. 2002; Ng & Low 2005; Borgeson et al. 2006; Azaza et al. 2009; Thompson et al. 2012).

Given their dietary flexibility, Tilapia culture offers the opportunity to utilize non-conventional feed resources. These include products not usually found on the market and items that are not considered traditional ingredients for commercial fish feed production. These products are usually non-competitive in terms of human consumption, cheap to purchase, and include items such as agricultural and aquacultural by-product or waste, plant waste, insects, and algae (Sogbesan and Ugwumba, 2008). Using non-conventional feed resources in Tilapia feed allows aquaculturalists to utilize locally available, non-competitive, and cost-effective resources.

1.8. Algae as an alternative

The FAO has earmarked single cell products, including algae, as potential alternatives to both fishmeal and terrestrial plant protein in fish diets (Habib et al. 2008). Proponents of algae claim that high protein algal species, such as Spirulina, are underutilized food resources for both humans and fed livestock (Habib et al. 2008). To date, food grade algae has found the most popularity as a protein supplement in the health food industry, and is included in fish feed only as an enhancer (<2%) (Mustafa and Nakagawa, 1995). There is evidence, however, to suggest that algal species have great potential as an effective fish feed supplement, and protein source (Habib et al. 2008). Spirulina species are the most frequently used (Mustafa and Nakagawa 1995), and have been specifically selected by the FAO as an area of interest given its complete nutritional profile (Habib et al. 2008).

1.8.1 Spirulina background

Spirulina is a filamentous, spiral shaped, multicellular cyanobacteria that thrives in warm, alkaline environments (Kebebe and Ahlgren, 1966). The largest natural sources of Spirulina are found in the Central Africa region around Lake Chad and Niger, in East Africa, along the Great Rift Valley, and in Mexico, where it has routinely been used as a human food source (Habib et al. 2008). Spirulina is also included in the diets of many fishes, including Tilapia (Khallaf and Alne-na-ei, 1987; Belay et al., 1996; Shimamatsu, 2004).

1.8.2 Nutritional information

Spirulina is a nutritionally balanced ingredient for inclusion in aquafeed (Table 1). It has a protein content ranging between 55-70% by dry weight (Phang et al. 2000) and contains all ten of the essential amino acids in appropriate levels, except for reduced methionine, cysteine, and lysine. Spirulina's essential fatty acid profile includes high amounts of poly-unsaturated fatty acids, and its vitamin and mineral contents are comparable to other in-use aquafeed protein sources.

Table 1) Nutritional profile of Spirulina in comparison to fishmeal

	Spirulina (Sarker et al. 2016)	**Brown fishmeal (Olvera-Novoa et al. 1998)**
Proximate composition (g 100g^{-1} as is)		
Dry matter	82.2	92.28
Crude protein	61.3	67.44
Ash	6.9	12.76
Lipid	5.5	10.52
Crude fibre	3.0	1.46
Essential amino acids (g 100g^{-1})		
Arginine	4.10	3.67
Lysine	3.10	1.55
Isoleucine	2.60	3.02
Leucine	4.70	4.84
Histidine	1.00	5.05
Methionine	1.37	1.87
Phenylalanine	2.50	2.69
Threonine	2.70	2.71
Tryptophan	1.20	0.72
Valine	0.30	3.41

1.8.3 Digestibility

Spirulina is comparable in digestibility to fishmeal (Sarker et al. 2016). Unlike other algal species, such as Chlorella, Spirulina lacks cellulose in its cell wall, which is known to decrease digestibility (Sarker et al. 2016). Fish that typically consume Spirulina, including Tilapia, have been found to have low stomach pH when full (approximately 1.4), which facilitates cyanobacteria lysis, and therefore increases digestibility (Moriarty, 1973). The literature does not suggest that poor Spirulina digestibility, especially in Tilapia, would limit nutrient absorption and growth.

1.8.4 Low inclusion levels

The literature concerning low levels of fishmeal replaced with Spirulina is uniformly positive. Between 0.5-10% Spirulina inclusion has led to positive growth results. For example, Parrotfish *(Oplegnathus fasciatus*) fed 5% Spirulina experienced significantly higher weight gain, protein efficiency ratios, feed intake, and lower feed conversion ratios than the fishmeal control (Kim et al. 2013). Similarly, Rainbow Trout (*Oncorhynchus mykiss*) experienced the highest weight gain when fishmeal was replaced with 7.5% Spirulina (Teimouri et al. 2013).

Low level Spirulina inclusion has been shown to impart a number of additional benefits. Spirulina has been reported to increase non-specific immune activity, and increase both juvenile and grow-out survival of many species when presented with specific pathogens (Yeganeh et al. 2015, Watanuki et al. 2006, Ibrahem et al. 2013). Spirulina inclusion can also increase fillet colour and firmness. Red Tilapia fed 30% *Spirulina platensis*, contained more carotenoids than individuals fed less Spirulina, demonstrating that *S. platensis* can be exploited as a pigment source for incorporation in

Red Tilapia diets to produce coloration and gain market acceptance (Ruangsomboon et al. 2010*).* Watanabe et al. (1990), reported improved taste, and texture of Striped Jack (*Pseudocaranx dentex*) related to depressed lipid content in muscle, when supplied with 5% dietary Spirulina. When Spirulina was included at 2%, Tilapia displayed increased muscle quality, increased firmness, and fibrousness of raw meat (Mustafa et al. 1994). Overall, growth, immune system response, colour, and taste of many fish species, including Tilapia, fed low levels of Spirulina improved.

1.8.5 High inclusion levels

The consequences of using Spirulina as a main protein source or in excess of 10% when replacing fishmeal are less clear. In many cases, growth results for diets containing over 10% Spirulina protein have been encouraging. Golden Barb (*Puntius gelius*) displayed significantly increased growth rates when 20% of the fishmeal in their diets was substituted for Spirulina (Hajiahmadian et al. 2012). Similarly, Silver Seabream (*Rhabdosargus sarba*) fed a diet containing up to 50% Spirulina did not suffer negative growth effects compared to a fishmeal control, and had a similar feed conversion ratio (FCR) (El Sayed, 1994). Tilapia have been fed diets containing 43% Spirulina without growth or feed intake being negatively impacted, and had a better FCR than a corn gluten meal control (Hussein et al. 2013). Therefore, growth results for both omnivorous and carnivorous fish fed diets containing Spirulina as a protein source have been significantly higher or comparable to those of the commercial or control diets.

Other studies, however, have found that high Spirulina inclusion results in depressed growth rates. Sharma and Panta (2012), and El Sayed (1994) suggest that Spirulina inclusion above 30% is detrimental to fish growth. Similarly, Olvera-Novoa et al. (1998) reported elevated growth rates in diets containing 10% and 20% Spirulina protein, but halved growth rates in diets containing more than 40% Spirulina compared to a fishmeal control. Given the nutritional composition of Spirulina and its relative digestibility, it should be a viable alternative protein source for use in aquafeeds provided that feed intake is maintained. Conflicting results in the literature suggests that more research evaluating the grow-out potential of Spirulina, and pinpointing factors affecting its effectiveness should be undertaken.

1.8.6 Spirulina availability and cost

At current market prices, Spirulina is unlikely to be incorporated into aquafeeds at high levels. The price of Spirulina is not well established but the FAO has quoted it to be approximately 1000 CAD/tonne. Spirulina's price likely reflects its use as a human health food supplement, and is marked up. Spirulina can be produced on a variety of substrates using exceptionally primitive or high tech methods. In fact, Spirulina can be cultivated in unlined ditches, harvested using suitable cloth material, and dried out in the sun (Habib et al., 2008). Chowdhury et al. (1995) suggest Spirulina costs approximately 0.5$/kg when cultured in a dug out trench. The price of purchasing Spirulina from an established supplier, and its production price are different, and will play a role in aquafeed incorporation.

1.9. Objective of the study

The purpose of this study was to evaluate the appropriateness of Spirulina and soybean meal as the sole protein sources in grow-out Tilapia diets. To achieve this goal, we designed 3 experimental diets with high levels of Spirulina (>10%) and soybean meal as their main protein sources. We compared these diets to two controls: a 100%

soybean meal protein diet, and a commercial Tilapia diet. Additionally, to evaluate the benefit of fishmeal inclusion, low levels (<10%) of fishmeal were added to the most successful Spirulina containing diet. We evaluated these experimental diets based on their physical stability, palatability, growth potential, waste production, and overall cost.

This study will address three major gaps in the literature. First, this study incorporated Spirulina into diets with no or low levels of fishmeal. Contemporary commercial Tilapia diets are mostly plant protein based and incorporate no more than 10% fishmeal. The control diets for many studies however, have used fishmeal in excess of what is typically employed by industry. For example, the control diets for Sheekh et al. (2014) and Velasquez et al. (2015) contained 25% and 56.5% fishmeal respectively. Similarly, the most successful growth promoting Spirulina diet for Olvera-Novoa et al. (1998) included over 41% fishmeal compared to 10.47% Spirulina. Hernandez et al. (2012) studied the total replacement of fishmeal with soybean meal and Spirulina, but in Rainbow Trout, not Tilapia.

Secondly, this study evaluated Tilapia grow-out diets. The literature typically focuses on fingerling Tilapia diets. Fingerlings require higher protein content diets, which makes them an important life stage to study, but the majority of aquafeed is administered to grow-out fish (20g- market size) which have largely been ignored (Lovell, 1998).

Lastly, to our knowledge, pellet stability, palatability (including the use of enhancers), waste production, and profitability have not been investigated in Tilapia diets containing high levels of Spirulina. The literature to date addresses the growth potential of Spirulina containing diets across several species, and only explores waste production in Rainbow Trout (Hernandez et al. 2012). Given this paucity of information, a study investigating all of the important factors to consider when evaluating a novel protein source should be conducted.

Ultimately, information regarding the stability, growth potential, palatability, waste products, and associated costs of Tilapia grow-out diets containing accurate levels of fishmeal and high levels of Spirulina is not available. This study aims to gather information about the growth potential of Spirulina containing diets, but also evaluate the effects Spirulina may have on a number of other important factors affecting novel aquafeed ingredient adoption which have otherwise been overlooked.

Chapter 2. Methods

2.1) Fish selection and husbandry

Experiments were conducted in the Aquatic Facility at Queen's University, Kingston, Canada. The fish used were bred and raised at the facility. Prior to the feeding trials, the fish were reared in recirculating tanks and fed a trout feed (Martin Profishent Classic Floating Fish Feed, Martin Mills, Guelph, Canada). Temperature in the recirculating system remained between 24.9 and 26.6 C° (H198311 DiST 5 EC/TDS/Temperature Tester, Hanna Instruments). The dissolved oxygen (DO) in the recirculating system remained between 69.7 and 88.0% (S110 Portable DO Monitor, Qubit Systems). For each experiment, fish were selected based on size and health. Due to their size at selection, we were unable to differentiate between male and female fish, and sex ratios in experimental tanks were not reported.

2.2) Diet formulation and preparation

Four experimental diets were formulated to contain 0 (SBM), 15 (SP15), 30 (SP30), and 45% (SP45) dry weight Spirulina spp. (Nuts.com, USA)(Table 2). A commercial tilapia diet (Tilapia Intermediate Pellet, Premium Fish Food, USA) was included as a commercial control (CC). The experimental diets were formulated to meet species requirements (FAO, website), with a minimum of 30% crude protein. The proximal composition of each diet was calculated by Agri-Food Laboratories (Guelph, Canada) and the essential amino acid composition of each feed was calculated according to available content information (Sarker et al. 2016) (Table 3).

Table 2) Composition of experimental diets (g 100g^{-1})

Ingredient	SBM	SP15	SP30	SP45
Soybean meal[1]	60	45	30	15
Spirulina[2]	0	15	30	45
Corn starch[3]	12	12	12	12
Flour[4]	20	20	20	20
Soybean oil[5]	6.3	6.3	6.3	6.3
Vitamin premix[6]	0.2	0.2	0.2	0.2
Mineral premix[7]	1.0	1.0	1.0	1.0
DL-methionine[8]	0.5	0.5	0.5	0.5
Total	100	100	100	100

1 Master Feeds, dehulled, heat treated, 47% crude protein 2 Nuts.com, USA 3 Selection Corn Starch, Metro, Canada 4 Five Roses All-Purpose Flour, Canada 5 Crisco Soybean Oil, Canada 6 DSM Nutritional Products Canada Inc. Vitamin premix as MG/Kg unless otherwise stated: Vitamin A (as Rovimix A 1000) 375000 IU/kg; Vitamin A (as Rovimix AD3 1000/200) 4 375 000 IU/kg; Vitamin D (as Rovimix AD3 1000/200) 875 000 IU/kg; Vitamin E (as Rovimix E-50 Adsorbate US) 100 000 IU/kg; Menadione (as MSBC) 3 750; Thiamine (as Rovimix B1) 7500, Riboflavin (as Rovimix B2 80-SD) 8 750, Pyridoxine (as Vitamin B6 Pyridoxine HCL) 10 000, Vitamin B12 (as Vitamin B12 1% Feed Grade) 17.5; Ascorbic acid (as Rovimix niacin) 50 000, Pantothenic acid (as calpan feed CN) 22 500; Folic acid (as Rovimix Folic 80 SD) 2 750; Biotin (Rovimix Biotin HP) 37.5, Calcium carbonate 50 000; Inositol 200 000; and Wheat Midds 136 326. 7 DSM Nutritional Products Canada Inc. Mineral premix as MG/Kg: Copper (as Copper sulphate 25%) 196; Iron (as Ferrous Sulphate 30%) 7 500; Manganese (as manganese sulphate 31.5%) 800; Cobalt (as cobalt sulphate 32% Co) 153; Iodine (as Calcium iodate 62%) 183; Zinc (as Zinc sulphate 35.5% PDR) 1 953; Magnesium (as Mag. Sul. 10-Epsom salts) 12 572; Sodium chloride 60 000; Potassium (as Dyna-K White) 25 750; monocalcium dicalcium phosphate (as Dical phosphate feed grade) 724 444; and Choline chloride (60%) 40 000. 8 99%, FCC, Sigma Aldrich

Table 3) Biochemical composition of the experimental diets (% diet)

Nutrient content per analysis[1]	Requirements[2]	SBM	SP15	SP30	SP45	CC
Moisture		11.3	12.35	11.68	11.16	6.07
Crude protein % (N x 6.25)		31.18	31.35	36.26	39.42	41.77

Fat %		5.46	6.65	6.63	7.22	7.97
Crude fibre %		2.78	2.26	1.91	0.97	2.91
Ash %		4.3	4.4	4.7	5.1	9.1
Carbohydrates % (calculated)		47.76	45.25	40.73	37.1	35.09
Calculated EAA content (g/100g diet as fed)						
Arginine	1.18	2.22	2.31	2.41	2.50	
Histidine	0.48	0.78	0.75	0.72	0.69	
Isoleucine	0.87	1.41	1.47	1.54	1.60	
Leucine	0.95	2.40	2.55	2.70	2.85	
Lysine	1.43	1.88	1.90	1.91	1.93	
Methionine	0.75	0.91	1.03	1.14	1.25	
Cysteine		0.50	0.49	0.48	0.47	
Phenylalanine	1.05	1.93	1.95	1.97	2.00	
Tyrosine		1.10	1.23	1.37	1.50	
Threonine	1.05	1.25	1.36	1.48	1.59	
Tryptophan	0.28	0.39	0.47	0.56	0.64	
Valine	0.78	1.58	1.26	0.94	0.63	

1 Analysis performed by SGS
2 Santiago and Lovell, 1998

To create the feed pellets, the relative proportions of dry ingredients were weighed and thoroughly mixed, before adding soybean oil and water. The mixture was then smeared across a 0.32 cm deep perforated plastic sheet with holes of 0.32 cm diameter and left to air-dry overnight. The pellets were stored in a cool and dry container until use.

Although all feeds were designed to be same size, the commercial control was larger than the experimental feeds. The dimensions of the feed fall within the acceptable range for the size of fish used in the following experiments (FAO, website2). All three Spirulina containing feeds had similar mean pellet weights (0.01350-0.01312g/ pellet. The soybean meal control was heavier (0.01478g ± 0.0025) than the Spirulina containing diets, and the commercial control was the heaviest of the feeds (0.02186g ± 0.00111). The commercial control was the least dense (525g/L) and the soybean meal and Spirulina diets have similar densities between 625-650 g/L. The commercial control pellets were the only ones able to float (Table 4).

Table 4) Mean density of experimental feed pellets

	Pellet weight (g)	**Pellet density (g/L)**	**Floating?**
CC	0.02186 (0.00111)	525	Yes
SBM	0.01478 (0.00025)	650	No
SP15	0.0135 (0.00018)	625	No
SP30	0.01312 (0.00039)	650	No
SP45	0.01312 (0.00025)	625	No

Values are means (se) of 5 replicates.

2.3) Feed stability testing

Individual pellets were weighed, and average density (g/L) determined. Five 500mL beakers were cleaned and filled with 250mL of 30C° water. Each beaker was assigned one feed. One gram of feed was added to the beaker for 5 minutes and removed. The following water stability indices were calculated (Effiong et al. 2009):

- Weight gain= initial weight- final weight
- Absorption efficiency rate (AER)= weight gain/time elapsed

- Relative absorption rate (RAR)= (final weight-initial weight)/initial weight

Whether or not the pellets floated was recorded. The experiments were repeated 5 times for 5 replicates.

2.4) Growth trials

The grow-out experiment testing Spirulina inclusion rates occurred in two blocks. In the first block, 110 grow-out fish (81.7 ±1.3g) were randomly selected from the recirculating system and stocked in one of 11 glass aquaria (136 ± 3.0L). Due to resource limitations, similarly sized fish could not be used for the second block. Instead, two different sizes of fish were used, and the number of fish in a tank was lowered to attain a similar tank density as the first block (6.7± 0.17g/L; 7.2± 0.20 g/L respectively). In the second block, 63 grow-out fish (122.5 ± 1.8 g) were randomly selected from the recirculating system and stocked in one of 9 glass aquaria (141.3 ± 3.7 L), and 12 grow-out fish (158.3 ± 3.6g) were randomly selected and stocked in one of 2 glass aquaria (118 ± 0L). Across the two experimental blocks, each feed treatment was assigned 3 or 5 replicates.

After one day of acclimation, the test diets were hand-fed to tanks *ad libitum* to a maximum of 1.5% body weight twice daily, six days a week (Friday to Wednesday). Feedings were performed in the morning and afternoon 7-8 hours apart. The amount of feed administered at each feeding was recorded. Each fish in each tank was weighed and measured weekly (after a minimum 12h starvation period), and the feeding rations were adjusted accordingly. Dead fish were not replaced during the experiment. Water quality was: dissolved oxygen 65.6- 91.6%, and temperature 26.2-28.4 C° with fluctuations occurring at approximately the same time in all tanks.

The feeding experiments lasted 4 weeks, at which point final weights and lengths were recorded before returning the fish to the holding tanks in the recirculating system. Growth performance was determined according to the following measures:

- Weight gain= ((final weight(g)- initial weight(g))/(initial weight(g)))*100%
- Daily weight gain= (weight gain (g)/28 days)
- Specific growth rate (SGR)= (ln final weight (g)-ln initial weight (g)/ number of days)
- Feed conversion rate (FCR)= dry feed intake (g)/ wet weight gain (g)

2.4.1) Waste product collection

Feed's basal total ammonia nitrogen (TAN) and total phosphorus (TP) levels were assessed by introducing 1.0g of each feed into a beaker of 200mL of water. After 36h, 10mL samples from the beakers were diluted by 100mL of fresh water. The mixture was agitated. TAN and TP samples were taken using the HI 715 Ammonia Medium Range Tester and HI 706 Phosphorus High Range Tester from Hanna Instruments.

Waste experiments were conducted simultaneously to growth experiments. Before the introduction of fish, the TAN and TP levels of each tank were recorded. Partial water changes occurred three times a week, full water changes occurred once a week, and TAN and TP data was collected before the third water change every week.

2.4.2) Fishmeal growth trials

Fishmeal (Atlantic Herring meal, Premier AG Resources Ltd.) was added to the SP15 diet at 2 and 10%, replacing soybean meal (FM2, FM10 respectively). The growth trial procedures follow the outline above. 120 grow-out fish (75.9 ±3.9g) were randomly selected from the recirculating system and stocked in one of 12 glass aquaria (122.6± 2.8L). Water quality was: dissolved oxygen 73.1-96.0 %, and temperature 25.4-28.3 C°. The same schedule for water changes and waste collection was kept.

2.5) Palatability and enhancers trials

We attempted to establish the relative palatabilities of each diet using self-feeders. The use of self-feeders for studying palatability in fish was validated by Adron et al. (1973) using Rainbow Trout (*Oncorhynchus mykiss*), and has since been used in a variety of species, including Tilapia (Fortes-Silva et al. 2011). Dueling self-feeder trials are, however, typically used to test nutritional wisdom- the ability of a fish to select a diet based on its nutritional requirements. Whereas nutritional wisdom trials last for more than 7 days and rely on the development of post-ingestive cues, palatability experiments last for less than a week and rely on pre-ingestive cues such as taste, odour, and feeder position (Montoya et al. 2011). Each of our experimental replicates lasted three days, to avoid fish selecting diets according to post-ingestive cues and to limit the effect of social hierarchies. Feeder position preference was controlled for by alternating feed position (front and back) for each replicate, and using self-feeders filled with the same feed to establish feeder position preference (Boujard and Le Gouvello, 1977).

Three separate palatability experiments took place. The first experiment tested levels of Spirulina inclusion (SBM, SP15, SP30, CC), the second tested the inclusion of 2% citric acid (CA) (>99.5% (Sigma Aldrich)) and dried basil leaves (DB)(Great Value, Canada) as feed enhancers to SP15 (replacing soybean meal), and the third tested fishmeal inclusion to SP15 (2% and 10% replacing soybean meal). Each combination of feed for each experiment was tested in 6 replicates.

Each palatability experiment followed the same procedure. Fish were introduced to the self-feeders (Figure 1) two weeks before the start of experiments in holding aquaria (Fortes-Silva et al. 2010). All holding aquaria successfully used the self-feeders as the only form of feed introduction, evident through both feeder emptying, and fish growth. Ten fish (27.2 ± 1.3g) were stocked in each of the 11 experimental tanks (136 ± 3.0L). Each tank was outfitted with a filter (Fluval C3 Power Filter), heater, and airstone.

Two self-feeders were suspended over the middle of each aquaria, one slightly forward, one slightly back. Each self-feeder was filled with 10% of the total aquarium biomass. The self-feeders holding the CC diets had to be outfitted with larger dispensing holes in order to accommodate the larger diameter of the CC feed. The hole:feed diameter ratio was held constant between the experimental and commercial control feeds. The amount of feed left in the self-feeder was measured twice daily: once in the morning, and once in the late afternoon. Additional feed was not added to self-feeders upon emptying.

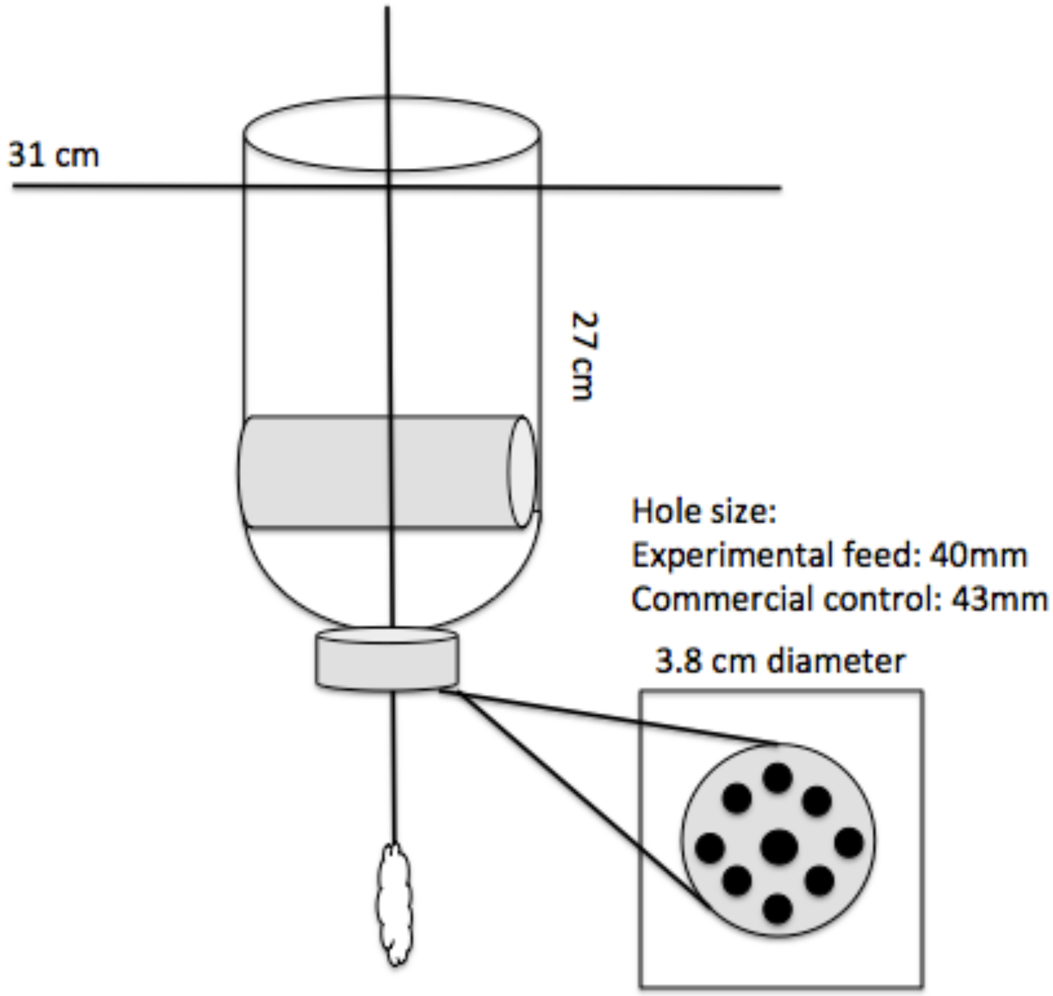


Figure 1) Self-feeder schematic (not to scale).

2.6) Statistical analysis

2.6.1) Stability statistics

The means of each measure of stability for the treatments were compared using a one-way analysis of variance (ANOVA). Significance was $p<0.05$. A post-hoc analysis was done when significant differences existed between treatment means using a Tukey's Honest Significant Difference Test. Analyses were performed using R (v.3.1.2). Before analysis, data were tested for normality.

2.6.2) Growth and waste statistics

ANOVAs were used to determine the differences between treatment means (Olvera-Novoa et al. 1998). Significance was $p<0.05$. A post-hoc analysis was done when significant differences existed between treatment means using a Tukey's Honest Significant Difference Test (Hernandez et al. 2012; El-Sayed Hussein et al. 2013).

Weight gain and waste results were assessed using linear model regressions. Initial models included relevant variables and were evaluated using the gvlma package for linear model assumptions ($p<0.05$). The initial model was dredged using the MuMIn package in R, and model averages were performed when more than one model had a delta<2.

2.6.3) Palatability statistics

The amount of feed dispensed by self-feeders between feeds within each feed combination was compared using paired t-tests (Xie et al. 2003, Montoya et al. 2011). Significance was $p<0.05$. The differences in feed dispensed between feeder positions were tested with paired t-tests with a significance of $p<0.05$.

Chapter 3. Results

3.1) Feed stability:

The largest water weight gain was by the commercial feed (CC) (101.0%), followed by SBM, SP15, SP30, and SP45. The absorption efficiency rate and relative absorption rate follow the same trend. The CC absorption efficiency rate (AER) and relative absorption rate (RAR) almost doubled those of SP45 (0.203 cm^3/min vs 0.114 cm^3/min; 100.97% vs 56.33%)(Table 5). The correlation between an experimental diet's Spirulina content and their relative absorption rate is -0.65, suggesting that Spirulina content has an inverse relationship with pellet water stability.

Table 5) Mean absorption rate of experimental feed pellets

	CC	SBM	SP15	SP30	SP45
Initial weight	1.003 (0.003)	1.005 (0.028)	1.010 (0.005)	1.003 (0.002)	1.011 (0.005)
Final weight	2.017 (0.052)	1.852 (0.053)	1.732 (0.046)	1.690 (0.033)	1.581 (0.082)
Weight gain (%)	101.0 (4.9)^{a}	84.3 (5.2)^{ab}	71.5 (4.8)^{bc}	57.1 (2.9)^{bc}	56.3 (7.9)^{c}
AER (cm^3/min)	0.203 (0.010)^{a}	0.169 (0.010)^{ab}	0.144 (0.009)^{bc}	0.137 (0.006)^{bc}	0.114 (0.062)^{c}
RAR (%)	100.97 (4.89)^{a}	84.31 (5.17) ^{ab}	71.52 (4.84)^{bc}	68.51 (2.91)^{bc}	56.33 (7.92)^{c}

Values in the same row with different subscripts are significantly different (p< 0.05).
Values are means (se) of five replicates.

3.2) Growth:

3.2.1) Spirulina feeds (SP15, SP30, SP45, SBM, and CC)

There were no statistical differences in growth performance between feed treatment groups (Table 6). The effect of feed type on growth and specific growth rate (SGR) was found to be close to significant (p=0.051; p=0.053 respectively). Upon further examination with a Tukey HSD post hoc analysis, no significant differences between diets were found. The fish fed the commercial control (CC) had the largest weight gain and SP15 (15% Spirulina) had the strongest growth and specific growth rate (SGR) among the experimental diets. Feed intake was the highest in SP15, followed by CC, SP30, SP45, and then SBM. Survival was above 85%, and deaths were unrelated to experimental treatment (the fish were bullied or jumped out of their tanks). Blocked ANOVAs revealed that there were significant differences between growth blocks in all measures except FCR (Appendix A2).

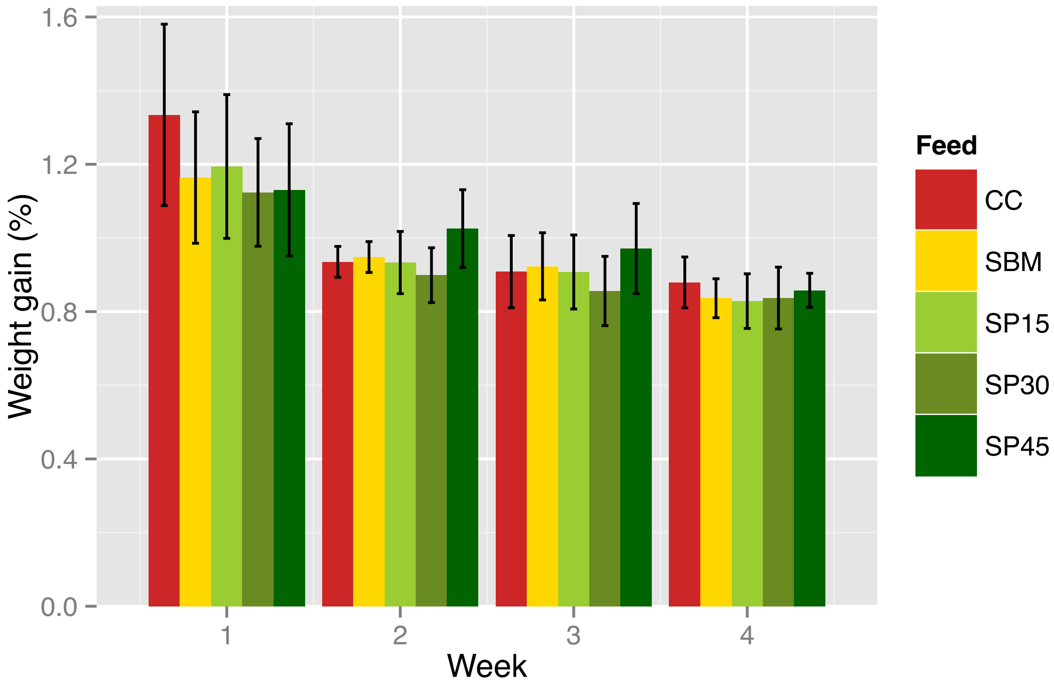
Table 6) The mean growth and feed intake of tilapia fed Spirulina and soybean meal based experimental diets for 4 weeks

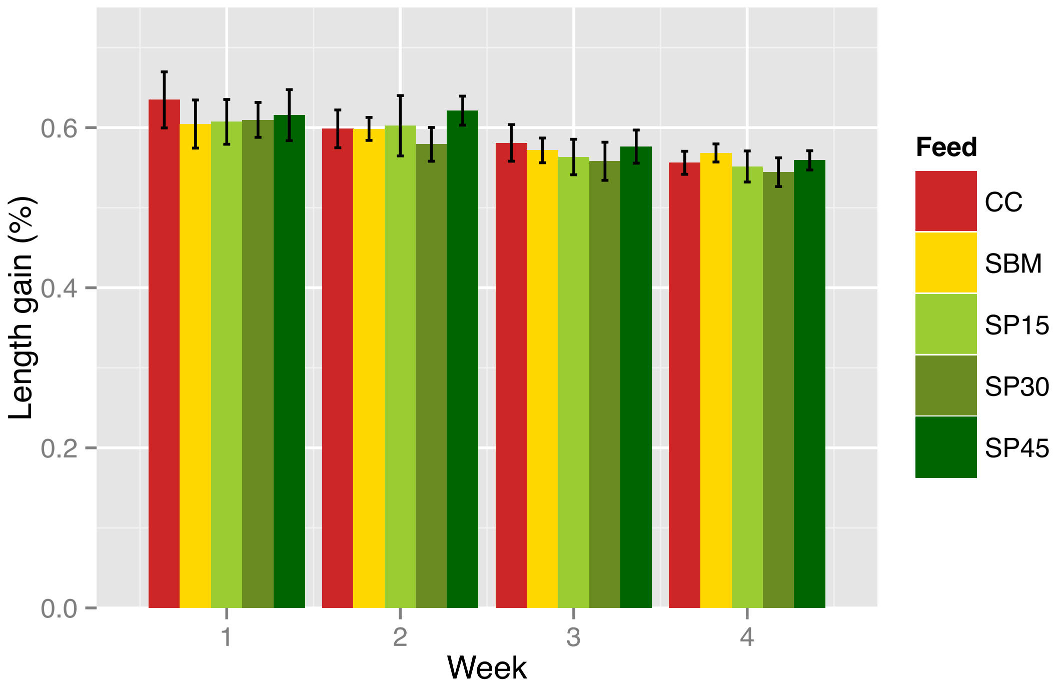
Mean values	CC	SBM	SP15	SP30	SP45
Survival (%)	100 (0.0)	87.9 (6.0)	92.9 (7.1)	96.7 (3.3)	85.4 (8.5)
Initial body weight	95.6 (13.6)	102.1 (11.3)	104.9 (15.2)	113.3 (14.7)	102.3 (12.7)
Final body weight	149.7 (3.6)	131.2 (6.1)	149.8 (11.7)	152.1 (12.7)	133.8 (5.2)
Weight gain (%)	54.5 (18.8)	28.1 (11.5)	40.4 (13.9)	27.0 (12.5)	27.8 (11.5)
Daily weight gain	1.95 (0.67)	1.00 (0.41)	1.44 (0.49)	0.96 (0.45)	0.99 (0.41)
SGR	1.69 (0.28)	1.02 (0.23)	1.29 (0.30)	0.99 (0.27)	0.93 (0.27)
Feed intake/day (% biomass)	2.18 (0.16)	1.86 (0.27)	2.28 (0.21)	2.15 (0.23)	1.99 (0.24)
FCR	1.27 (0.29)	0.91 (1.11)	0.76 (0.85)	1.14 (1.01)	2.52 (0.67)

Values are means (se) of between 3-5 replicates

Through a linear regression model including the variables feed treatment, percent feed intake, initial weight, and block, it was confirmed that the best model to predict weight gain included percent feed intake and initial average weight of fish (AICc=268.3, delta= 0.00, df=4). A model average (delta<2) included percent feed intake, initial average weight, and block.

The fish increased in weight and length each week for all feeds (Figure 2). Feed intake (g feed/g total tank biomass) peaked in week 2 for every feed except for SP30 (peaked in week 3). SP15 consistently exhibited the highest feed intake value among experimental feeds, except in week 3, when SP30 peaked. SBM exhibited the lowest feed intake value every week.





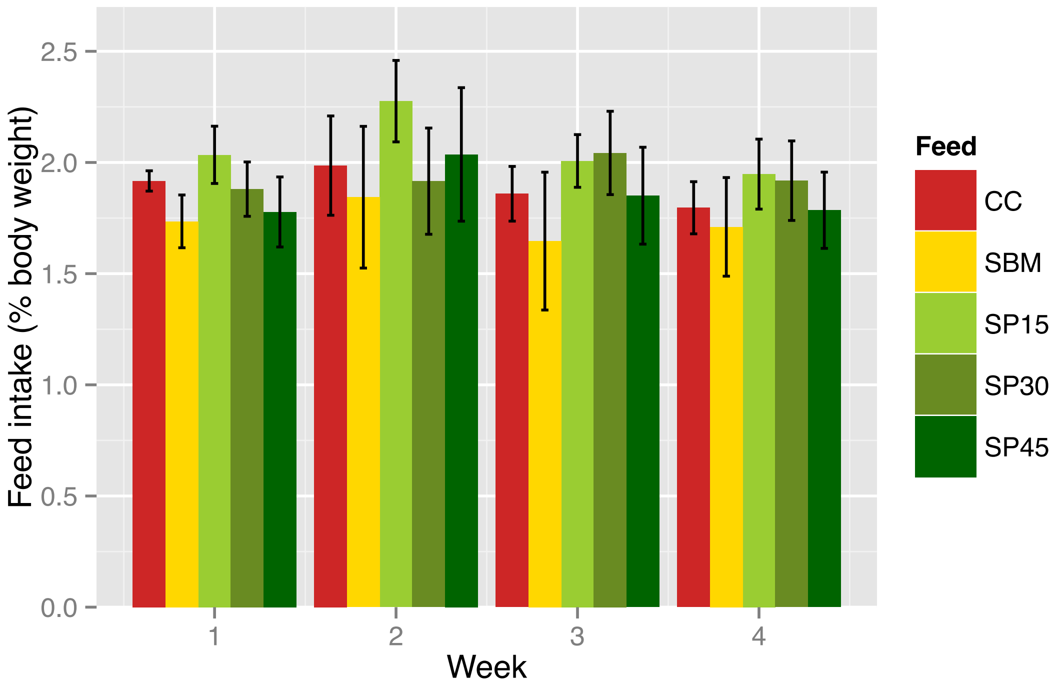


Figure 2) Mean tilapia weight gain (%), length gain (%), and feed intake (% body weight) (mean ± standard error) plotted over the duration of the study for Spirulina diet experiment (n=3-5).

3.2.2) Fishmeal feeds (FM2, FM10, SP15, and CC)

There were no statistical differences in growth performance between feed treatments (Table 7). There were no mortalities. Fish fed FM2 had the largest weight gain and the strongest SGR. Feed intake was the highest in SP15 and FM2, followed by FM10 and CC.

Table 7) The mean growth and feed intake of Tilapia fed Spirulina, soybean meal, and fishmeal based experimental diets for 4 weeks

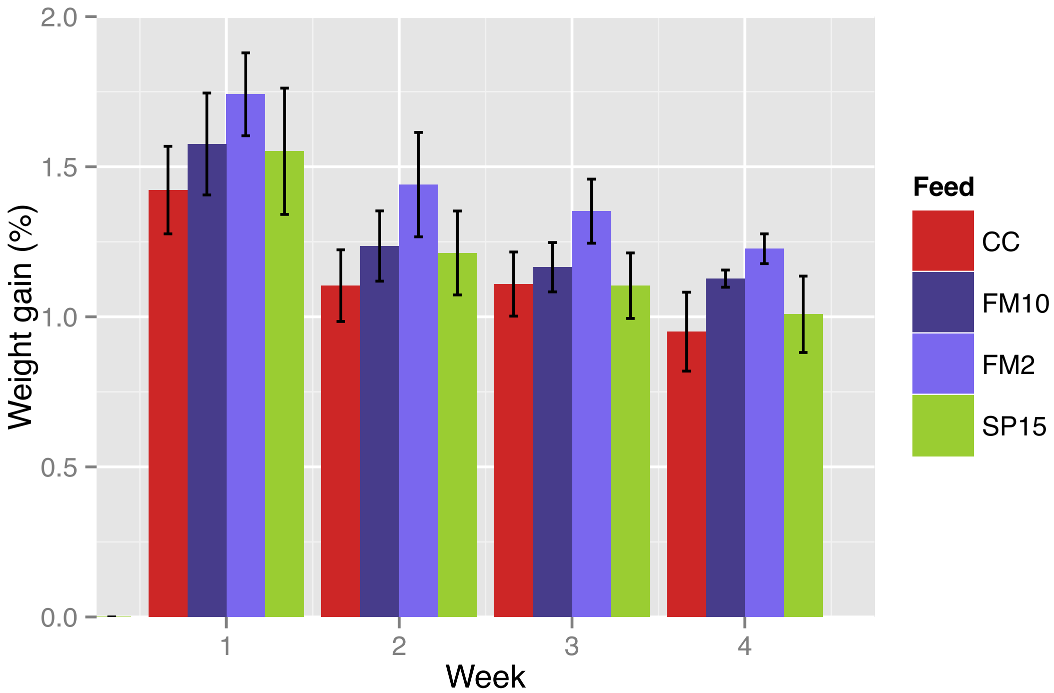
Mean values	CC	SP15	FM2	FM10
Survival (%)	100	100	100	100
Initial body weight	83.6 (9.0)	78.7 (4.4)	66.6 (5.2)	74.8 (6.1)
Final body weight	117.9 (17.3)	117.5 (17.5)	99.0 (4.7)	102.7 (2.8)
Weight gain (%)	40.0 (5.3)	48.6 (5.8)	49.3 (4.9)	38.5 (8.2)
Daily weight gain	1.43 (0.19)	1.74 (0.21)	1.76 (0.17)	1.38 (0.29)
Specific Growth Rate	1.20 (0.13)	1.41 (0.14)	1.43 (0.12)	1.15 (0.21)

Feed intake/day (% biomass)	1.68 (0.07)	2.17 (0.17)	2.17 (0.23)	1.86 (0.17)
FCR	1.25 (0.10)	1.36 (0.10)	1.29 (0.04)	1.47 (0.12)

Values are means (se) of 3 replicates.

A linear regression model confirmed that percent feed intake was the only significant predictor of weight gain ($p<0.001$). Percent feed intake and initial average weight of fish are included in the best model to predict weight gain (AICc=81.0, delta= 0.00, df=4).

The fish increased in weight and length each week for all feeds (Figure 3). SP15 and FM2 had the highest feed intake values across all four weeks, followed by FM10 and CC.



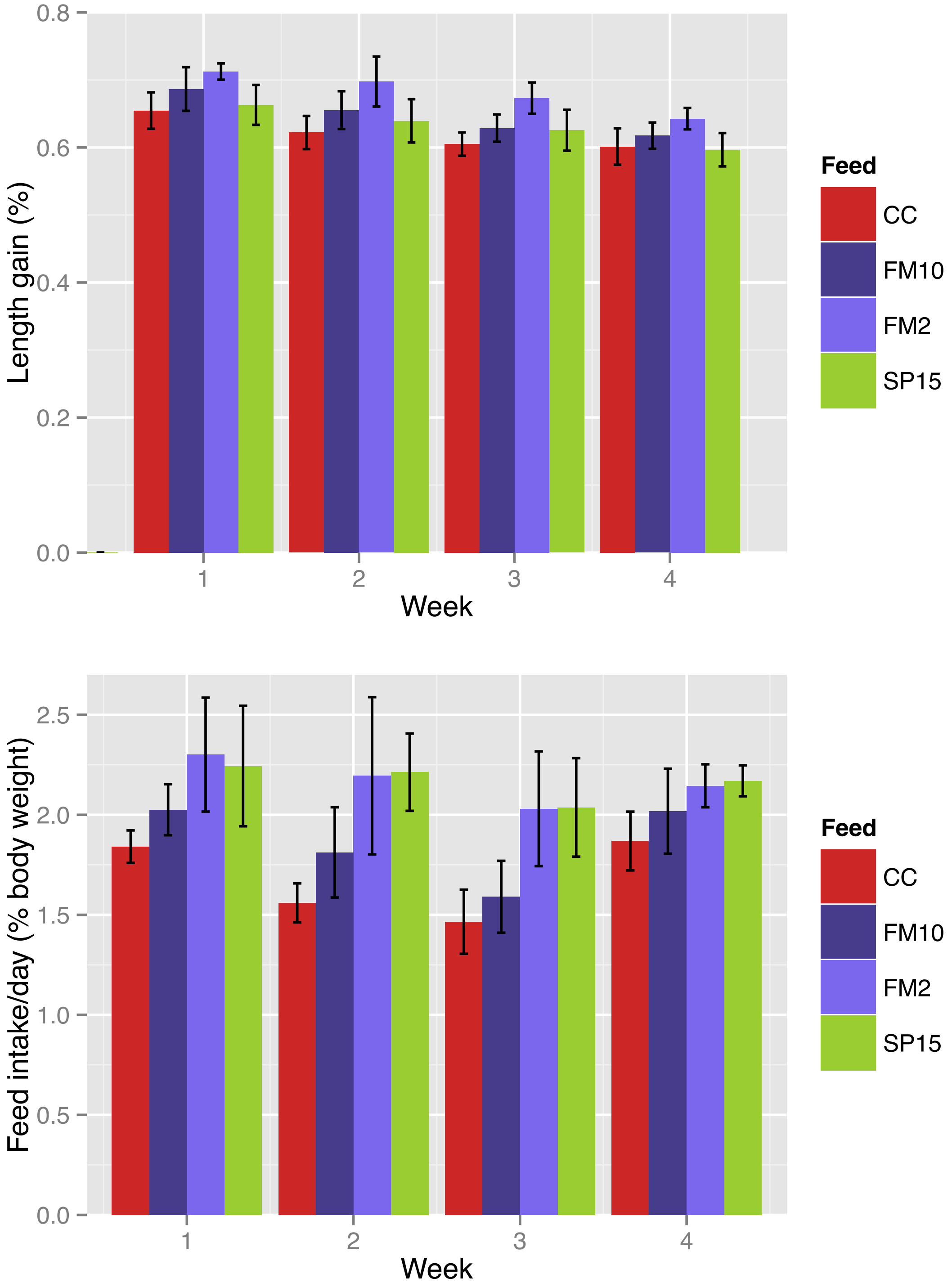


Figure 3) Mean tilapia weight gain (%), length gain (%) and feed intake (% body weight) (mean ± standard error) plotted over the duration of the study for fishmeal diet experiment (n=3).

3.3) Waste

CC had the highest TAN and TP. TAN and TP increased with increased Spirulina content. FM2 and FM10 had the same TAN levels as SP15, and the same or increased TP. SBM had the lowest TAN and TP (Table 8).

Table 8) Basal total ammonia nitrogen and total phosphorus levels for all diets.

	TAN (ppm)	TP (ppm)
SP15	0.0	0.9
SP30	0.18	1.2
SP45	0.20	1.5
FM2	0.0	0.9
FM10	0.0	1.7
CC	0.56	2.1
SBM	0.0	0.2

3.3.1) Waste Spirulina diets (SP15, SP30, SP45, SBM, and CC)

Total ammonia nitrogen (TAN) and total phosphorus (TP) levels in each tank were significantly affected by diet ($p<0.05$)(Table 9). Tanks administered CC had significantly higher TP levels than the experimental diets, and significantly lower TAN than tanks administered SP15. SP15 fed tanks had the highest TAN values. Blocks had significantly different TAN but not TP (Appendix A3).

Table 9) Total ammonia nitrogen and total phosphorus levels in tanks for Spirulina diet experiment.

	CC	SBM	SP15	SP30	SP45
Total ammonia nitrogen (ppm)	1.76 (0.86)^{a}	2.14 (1.23)^{ab}	4.86 (1.00)^{b}	3.72 (1.00)^{ab}	2.41 (1.22)^{ab}
Total phosphorus (ppm)	0.25 (0.04)^{a}	0.02 (0.01)^{b}	0.05 (0.01)^{bc}	0.10 (0.02)^{cd}	0.15 (0.01)^{d}

Values in the same row with different subscripts are significantly different ($p< 0.05$). Values are means (se) of at least 3 replicates.

Throughout the study, across all treatments, aquarium water TP levels were between 0.0-0.4 ppm and TAN levels were between 0.0-10.3 ppm (Figure 4). Across all diets except CC, week 4 ammonia levels were lower than week 1. TP levels remained steady between weeks 2 and 4.

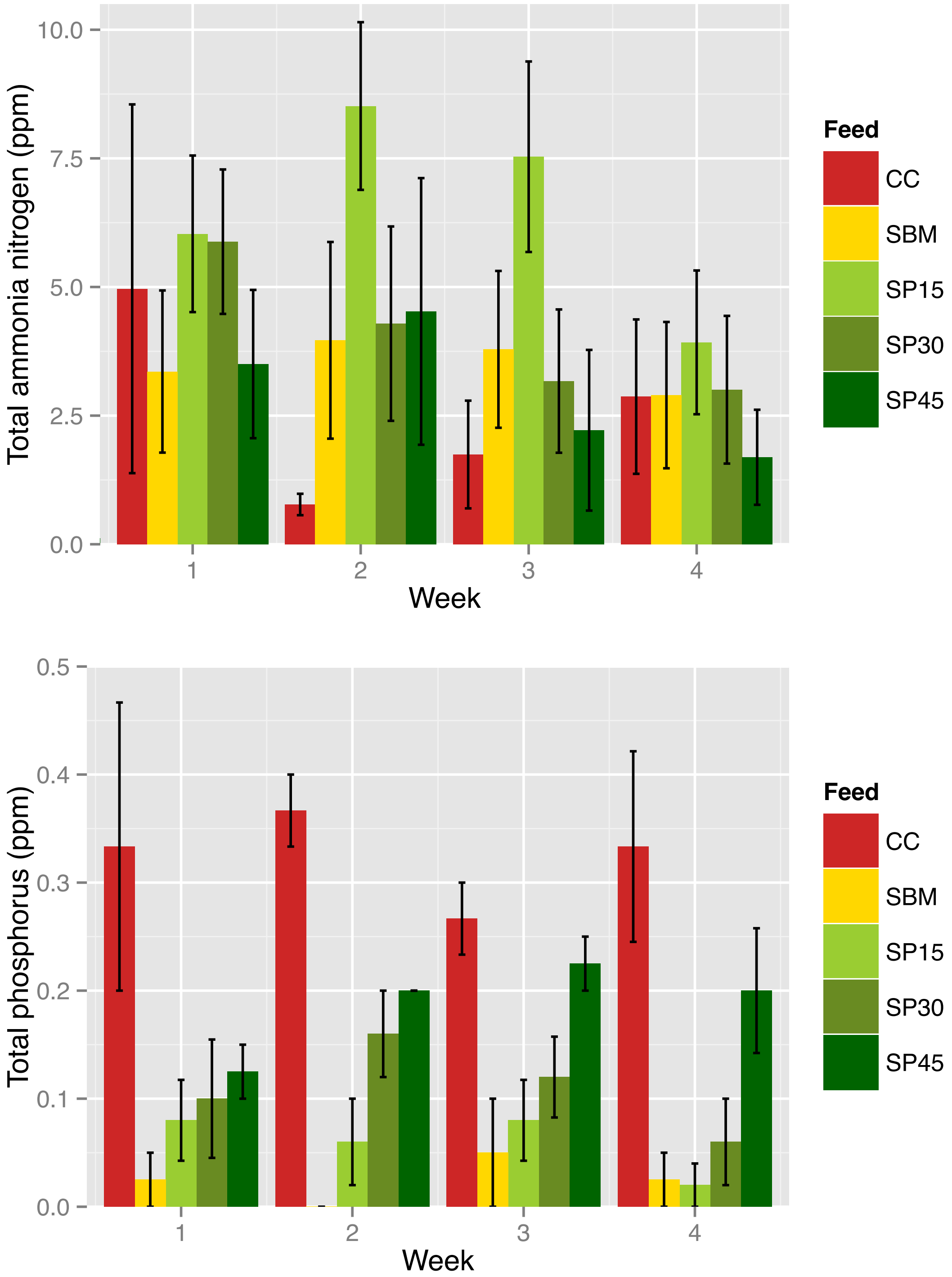
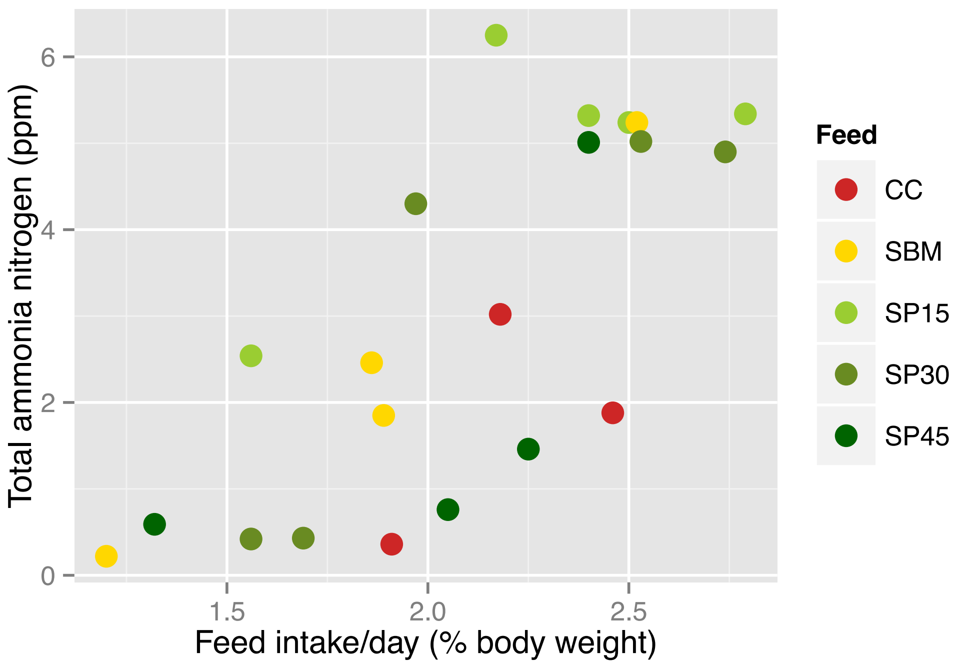


Figure 4) Mean total ammonia nitrogen (ppm) and total phosphorus (ppm) levels in aquarium water for Spirulina diet experiment over 4 weeks (mean ± standard error)(n=3-5).

The predictive power of total feed intake and feed treatment on TAN and TP levels was assessed. Feed intake is the most important predictor of TAN levels (R^2= 0.56) and feed type is the most important predictor of TP levels (R^2= 0.91)(Figure 5). We used a linear model including feed treatment, fish growth, and feed intake to predict TAN. The model was dredged (using the MuMIn package in R). The best model included only feed intake (AICc= 84.7, delta=0.00, df=3). The model average (delta<2) included feed intake, and growth (importance= 1.0, and 0.37 respectively). The same variables were used to predict TP. The best model after dredging, and the only model with a delta<2, included feed type and growth (AICc= -68.3, delta=0.00, df=7).



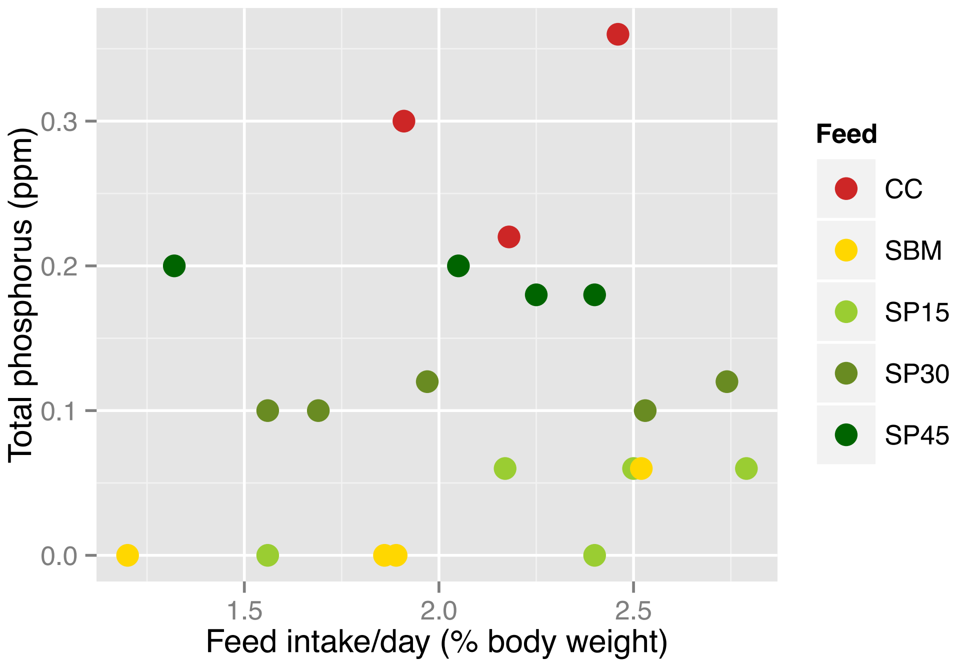


Figure 5) Feed intake as a predictor of total ammonia nitrogen (ppm) and total phosphorus (ppm) levels in aquaria water for Spirulina diet experiment (n=3-5).

3.3.2) Waste production in fishmeal containing diets (FM2, FM10, SP15, and CC)

TAN and TP levels in each tank were significantly affected by diet ($p<0.05$) (Table 10). Tanks administered CC had significantly higher TP levels than the experimental diets. SP15 had significantly higher TAN than FM2, FM10, and CC.

Table 10) Total ammonia nitrogen and total phosphorus levels in tanks for fishmeal diet experiment.

	CC	SP15	FM2	FM10
Total ammonia nitrogen (ppm)	0.75 (0.33)^{a}	3.64 (0.72)^{b}	1.72 (0.41)^{a}	1.17 (0.36)^{a}
Total phosphorus (ppm)	0.38 (0.07)^{a}	0.07 (0.02)^{b}	0.10 (0.03)^{b}	0.11 (0.02)^{b}

Values in the same row with different subscripts are significantly different ($p< 0.05$). Values are means (se) of 3 replicates.

Throughout the study, across all treatments, aquarium water TP levels were between 0.0-1.0 ppm and TAN levels were between 0.0-9.68 ppm (Figure 6). Week 4 TAN levels peaked and TP levels were relatively stable by week 2.

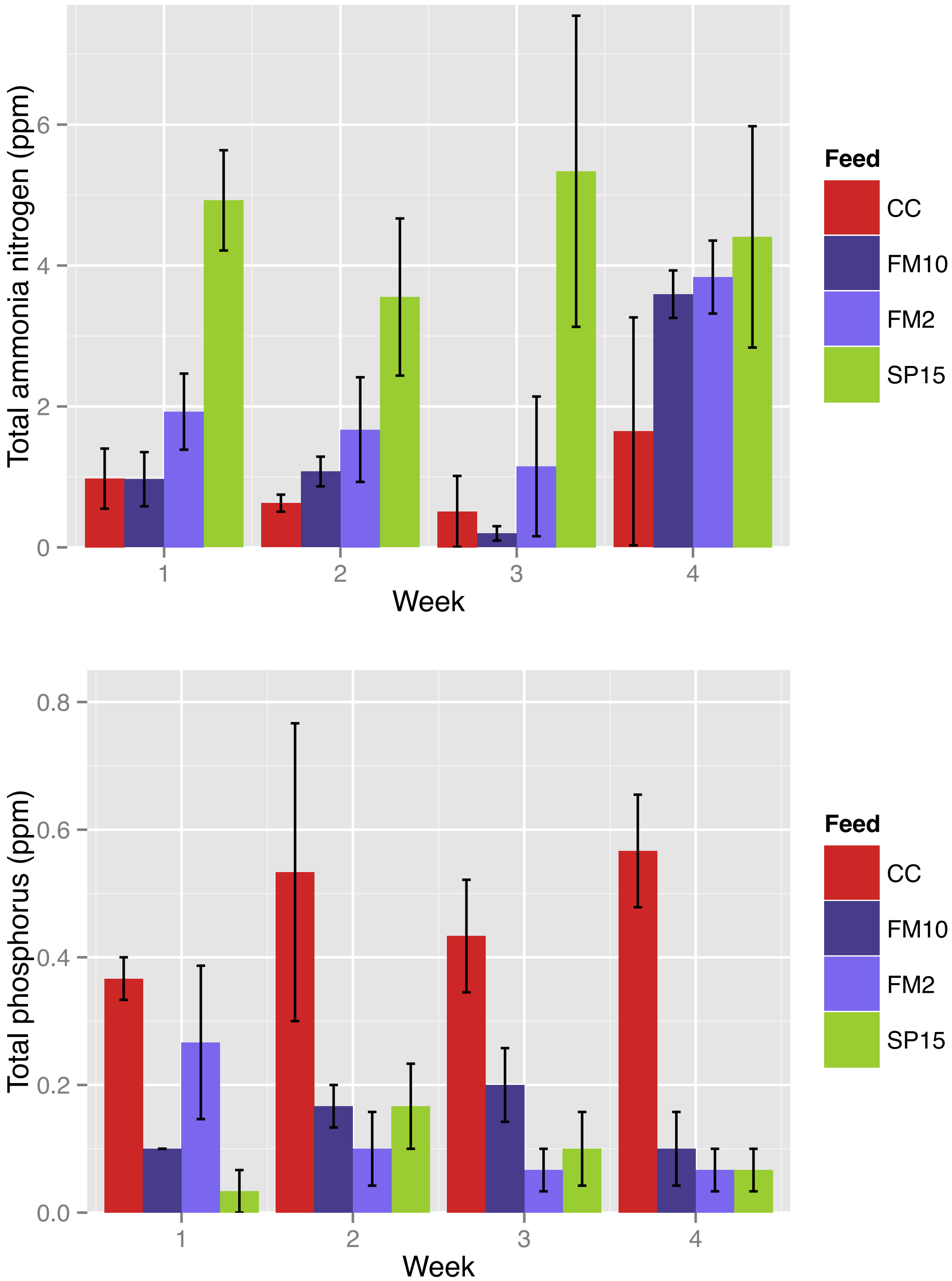
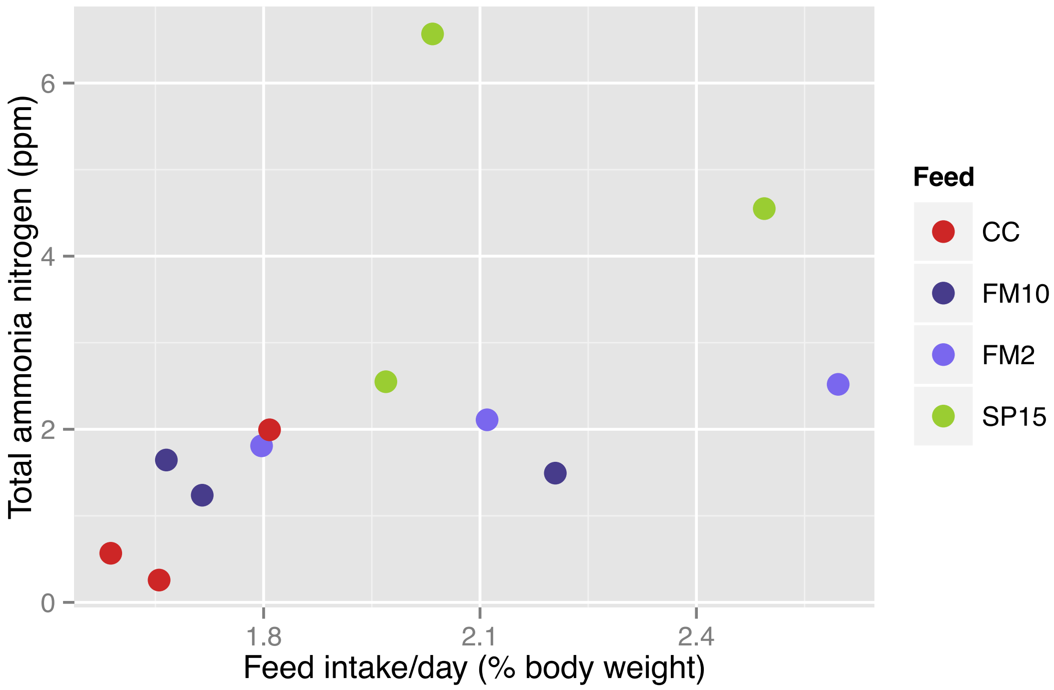


Figure 6) Mean total ammonia nitrogen (ppm) and total phosphorus (ppm) levels in aquarium water for fishmeal diet experiment over 4 weeks (mean ± standard error)(n=3).

We used a linear model including feed type, growth, and feed intake to predict TAN (R^2=0.82). The best model after dredging included only growth (AICc= 51.3, delta= 0.00, df=3). The model average (delta<2) feed type, growth, and percent feed intake (importance: 0.20, 0.29, 0.26 respectively). The same variables were used to predict TP. The best model only included feed type (AICc=7.5, delta= 0.00, df=5) (R^2=0.80). A model average of models included feed type, growth, and percent feed intake (importance 0.7, 0.3, and 0.3 respectively)(Figure 7).



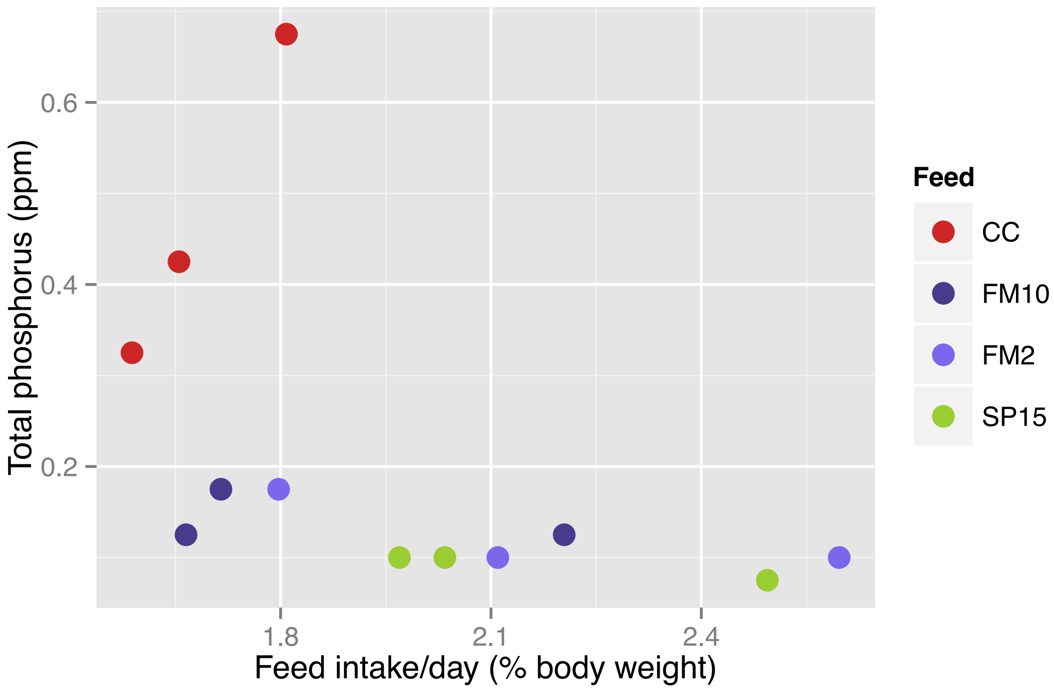


Figure 7) Feed intake (% body weight) as a predictor of total ammonia nitrogen (ppm) and total phosphorus (ppm) levels in aquaria water for fishmeal diet experiment (n=3).

3.4) Palatability

3.4.1) Spirulina diets (SP15, SP30, SP45, SBM, and CC)

Across all feed combinations, there was no significant difference between the mean amount of feed distributed from front located self-feeders and back self-feeders (5.32±0.41; 4.27±0.42 respectively). When self-feeders containing the same feed were compared, front located feeders were significantly ($p<0.05$) preferred (5.78± 0.65; 3.42± 0.63 respectively). When self-feed containing different feeds were compared, there was no significant difference between front and back-located self-feeders (5.01± 0.53; 4.83± 0.55 respectively).

A greater amount of the commercial diet (CC) was released from self-feeders compared to the experimental diets ($p<0.05$). No significant differences were detected among any of the other feed combinations ($p>0.05$) (Table 11). SP15 was preferred over

SP30, and was preferred over SBM. SBM was preferred over SP30, and SP30 was not preferred over any other feed.

Table 11) Feed preference for Spirulina feeds and controls

Combination	Difference (% body weight)	Preferred feed
CC-SP15	5.3 (1.36)	CC*
CC-SP30	7.2 (0.69)	CC**
CC-SBM	5.4(0.92)	CC**
SP15-SP30	4.8(1.35)	SP15
SP15-SBM	3.0(0.48)	SBM
SP30-SBM	4.9(1.21)	SBM

Values are means (se) of 6 replicates.
* $p < 0.05$; ** $p < 0.01$

3.4.2) Enhanced diets (SP15, DB, and CA)

It was observed that front located self-feeders were significantly ($p<0.05$) preferred over back feeders (2.29±0.31; 1.32±0.20 respectively). When self-feeders containing the same feed were compared, there was a significant difference ($p<0.05$) between the amount of feed distributed from front and back self-feeders (2.80± 0.42; 1.15± 0.42 respectively). When self-feed containing different feeds were compared, there was no significant difference between front and back-located self-feeders (1.67 ± 0.42; 1.52 ± 0.35 respectively).

The addition of dried basil (DB) and citric acid (CA) did not have a stimulatory effect on the amount of feed released from the self-feeders (Table 12). No significant differences were detected among enhanced feed combinations ($p>0.05$).

Table 12) Feed preference for enhanced feeds

Combination	Difference (% body weight)	Preferred feed
CA-SP15	1.8 (0.86)	SP15
DB-SP15	2.1 (0.64)	DB
CA-DB	2.2 (0.56)	CA

Values are means (se) of 6 replicates.

3.4.3) Fishmeal diets (FM2, FM10, SP15, and CC)

It was observed that there was no significant difference between the mean amount of feed distributed from front located self-feeders and back self-feeders (1.61±0.23; 1.63±0.28 respectively). When self-feeders containing the same and different feeds were compared, there was no significant difference between the amount of feed distributed from front and back self-feeders (1.70± 0.30 vs 1.49± 0.37; 1.51 ± 0.36; 1.77 ± 0.43 respectively).

The different test concentrations of fishmeal did not have a stimulatory effect on tilapia feed demand. No significant differences were detected among different feed combinations (p>0.05) (Table 13). F2 and SP15 were preferred over FM10.

Table 13) Feed preference for fishmeal diets

Combination	Difference (% body weight)	Preferred feed
SP15- FM2	2.2 (0.58)	FM2
SP15- FM10	1.8 (0.89)	SP15
FM2- FM10	2.3 (0.97)	FM2

Values are means (se) of 6 replicates.

3.5) Cost-benefit analysis

Fishmeal, soybean meal, and Spirulina prices were collected from various sources including the price paid for the Spirulina used in the experiment (Nuts.com)(Table 14).

Table 14) Farm gate price of protein products

	Cost of feed CAN$/t (source)
Fishmeal	2 328.03[1]
Soybean meal	425.25[1]
Spirulina	500[2] 1000[3] 7 151[4] 26 430[5]

1 IndexMundi; 2 Chowdhury et al. 1995; 3 FAO; 4 Beneman, 2008; 5 Nuts.com

The feed cost to produce one kilogram of fish (incidence cost) increased with increased Spirulina inclusion and price. The profit index for SP15 exceeded that of SBM across all prices of Spirulina except the most expensive. The profit for SP30 only exceeded that of SBM with Spirulina prices up to 1000$/t. SP45 did not outperform SBM at any price for Spirulina (Table 15).

Table 15) Cost benefit analysis of Spirulina diets (SP15, SP30, SP45, and SBM)

Spirulina price	Feed	Cost per kg feed	Change %	Incidence cost	Change %	Profit index	Change %
	SBM	0.26	100.00	6.22	100.00	7.17	100.00
Spirulina 500$/t	SP15	0.27	104.39	0.23	3.63	42.16	588.28
	SP30	0.28	108.79	0.59	9.47	12.44	173.51
	SP45	0.29	113.18	0.34	5.42	6.57	91.73
Spirulina 1000$/t	SP15	0.34	133.79	0.29	4.65	32.90	459.03
	SP30	0.43	167.58	0.91	14.59	8.07	112.64
	SP45	0.51	201.37	3.15	50.65	3.70	51.56
Spirulina 7151$/t	SP15	1.26	495.00	1.07	17.20	8.89	123.97
	SP30	2.27	891.00	4.82	77.60	1.52	21.19
	SP45	3.28	1286.00	20.11	323.54	0.58	0.58
Spirulina 26430$/t	SP15	4.16	1628.79	3.52	56.61	2.70	37.70
	SP30	8.06	3157.58	17.10	275.00	0.43	5.98
	SP45	11.96	4686.38	73.30	1178.85	0.16	2.22

Incidence cost= feed cost consumed/ kg fish produced
Profit index= value of fish crop/cost of feed consumed

The feed cost to produce one kilogram of fish (incidence cost) increased with increased fishmeal inclusion and increased Spirulina price. The profit index for SP15 exceeded that of FM2 across all prices of Spirulina up to 26 430$/t. FM10 did not outperform SP15 at any price for Spirulina (Table 16).

Table 16) Cost-benefit analysis of fishmeal diets (SP15, FM2, and FM10)

Spirulina price		Cost per kg feed	Change %	Incidence cost	Change %	Profit index	Change %
Spirulina 500$/t	SP15	0.27	100.00	0.15	100.00	20.39	100.00
	FM2	0.30	100.00	0.25	168.47	18.26	89.57
	FM10	1.08	100.00	1.08	723.46	4.59	22.52
Spirulina 1000$/t	SP15	0.34	100.00	0.19	100.00	15.91	100.00
	FM2	0.38	111.15	0.21	111.15	14.65	92.10
	FM10	0.53	155.74	0.30	155.74	9.35	58.79

Spirulina 7 151$/t	SP15	1.26	100.00	1.67	100.00	4.30	100.00
	FM2	1.30	103.17	1.68	100.63	4.27	99.38
	FM10	1.45	115.08	2.10	125.66	3.42	79.58
Spirulina 26 430$/t	SP15	4.16	100.00	2.32	100.00	1.31	100.00
	FM2	4.19	100.92	2.34	100.92	1.33	101.44
	FM10	4.27	102.68	2.38	102.68	1.17	89.17

Incidence cost= feed cost consumed/ kg fish produced
Profit index= value of fish crop/cost of feed consumed

Chapter 4. Discussion

This study analyzed the stability, growth potential, waste generation, palatability, and profitability of grow-out Nile Tilapia feeds containing Spirulina, soybean meal, and fishmeal**.** Spirulina inclusion decreased feed water stability, had no significant effect on growth performance measures or palatability, and decreased profitability. SP15 (15% Spirulina) tended to be the most effective Spirulina containing diet in terms of its stability, growth promotion, total phosphorus waste production, palatability, and cost effectiveness. Diets containing 30 and 45% Spirulina exhibited significantly decreased water stability, and tended to exhibit decreased feed intake and growth, increased total phosphorus production, decreased palatability, and decreased profitability compared to SP15, the soybean meal control, and the commercial control. The addition of fishmeal to SP15 did not significantly alter growth performance measures, waste production, or palatability. This study suggest that fishmeal can be removed from grow-out Nile Tilapia diets in favour of soybean meal and Spirulina while maximizing profitability and without compromising growth, if Spirulina content is limited to 15%.

Spirulina inclusion was found to have an inverse relationship with water stability indices. All three Spirulina containing diets were significantly less stable than CC, and SP45 was significantly less stable than SBM. Spirulina inclusion in the experimental diets therefore decreased stability over soybean meal inclusion.

While Spirulina addition decreased pellet stability compared to soybean meal, Hashim and Mat Saat (1992) observed that the addition of macro algae meal improved stability indices when compared against wheat flour, a common binding agent (Hashim and Mat Saat, 1992). Whereas Spirulina is a micro algae with a high protein content, macro algae are more likely to have high starch and non-starch polysaccharide contents which are responsible for providing the bonds necessary for high feed stability (Riaz, 1997). In sacrificing soybean meal for Spirulina, the protein content of the diets increased, but the crude fibre and carbohydrate content decreased (Table 3), representing the loss of binding materials. Given Spirulina's proximate composition, Spirulina containing diets may require added binding agents, such as guar gum or carrageenan, in order to maintain high stability levels.

Differences in protein and carbohydrate contents do not explain the similar stability indices between SBM and CC. Whereas CC contains 41.77% protein and 35.09% carbohydrates, SBM contains 31.18% protein and 47.76% carbohydrates, and the stability indices of these two diets did not differ significantly. This result may be explained by the different manufacturing methods used to create the diets. While SBM was manufactured through the procedures outlined in the methods section, CC was manufactured through a process known as extrusion. Extrusion has been observed to increase water stability over other feed pellet making techniques (Hilton et al. 1981), which likely explains the stability of CC despite its low carbohydrate content. If the experimental diets had been extruded, their stability measure would have also likely increased. That being said, carbohydrate content remains important for the extrusion process, as it is the gelatinization of starches by heat that causes much of the binding. It is unclear how much extrusion would have affected experimental diet stability.

Water stability is of the utmost importance for aquafeeds as it is a measure of a pellet's ability to avoid leaching water-fouling nutrients, and deliver maximum quantities of feed nutrients to a fed species (Hashim and Mat Saat, 1992). During the stability testing and upon feed introduction in the grow-out tanks, SP15, SP30, and SP45 stained the water a blue-green colour, suggesting that the pellets were leaching Spirulina. Finely ground materials are more likely to dissipate once the pellet starts disintegrating (Jayaram and Shetty, 1981), and Spirulina and the vitamin and mineral mixes were the most finely ground materials in the experimental feeds. Leaching was aggravated when Tilapia were feeding. Unlike many other fish species, Tilapia tend to "chew" pellets instead of swallowing them whole. Acceptable pellets will often be kept in their mouths for an extended period of time before swallowing and unacceptable pellets are often taken into the mouth and rejected several times before ultimately being discarded (Lovell, 1998). The feeding habits of Tilapia therefore necessitate stable feed pellets. The observed water staining is additional evidence that the Spirulina pellets were not stable and Spirulina's physical qualities combined with Tilapia feeding habits may have led to increased feed disintegration and therefore increased nutrient leaching and lost growth potential.

Each experimental unit demonstrated weight and length gains each week, suggesting that all of the tested diets promoted growth. Overall, there were no significant differences in growth performance measures between diets but strong trends were present; the p-value for a blocked ANOVA analyzing the effect of feed on growth and SGR approached the significance threshold of 0.05 (p=0.0503). CC and SP15 outperformed the other diets in each growth performance category. For example, SP15 and CC growth was approximately 50% higher than SP30, SP45, and SBM growth (40.4, 54.5, 27.0, 27.8, 28.1% respectively). This suggests that a 15% cap on Spirulina inclusion may result in the largest weight gain by grow-out tilapia behind a commercial control.

The results of this study, which evaluated Spirulina inclusion in grow-out Nile Tilapia, and the findings of some of the existing literature reviewing Spirulina inclusion in juvenile Tilapia diets, suggest that Spirulina inclusion should be limited. Velasquez et al. (2015) and Olvera Novoa et al. (1998) found that diets containing between 10-20%

Spirulina performed best, and that inclusion past 20% led to a 50% decrease in weight gain. It was suggested that the observed decline in growth from substituting fishmeal with Spirulina might stem from a decrease in protein quality (Olvera-Novoa et al. 1998). Olvera-Novoa et al. (1998) replaced soybean meal, a protein of lower quality, with Spirulina, a protein of higher quality with no known presence of anti-nutritional factors (Richmond, 1988), and observed a similar decrease in weight gain with increased Spirulina. Other studies similarly suggest that Spirulina protein is sufficient to promote growth. Hussein et al. (2013) observed significantly increased growth in a diet containing solely Spirulina protein (43.63%) over a control consisting of only corn gluten meal protein (43.35%) in Nile Tilapia larvae diets. Additionally, when replacing soybean meal with 0, 15, 30, and 45% Spirulina in a Rainbow Trout diet, a species less tolerant to low quality plant protein, Hernandez et al. (2012) did not observe any significant differences in growth. There was a trend, however, towards increased weight gain in fish fed increased levels of Spirulina, and the highest weight gain was by the fish fed the 60% fishmeal control diet (Hernandez et al. 2012). These results suggest that the protein quality of Spirulina may not be the factor limiting high levels of inclusion and this issue probably warrants further investigation.

Coupled with a decrease in growth, we observed a decrease in feed intake as Spirulina content increased past 15%. The two diets with the highest weight gain (CC and SP15) also experienced the highest feed intake (2.18 and 2.28 %biomass/day). Feed intake decreased as Spirulina inclusion increased, and feed intake for SBM was expectedly low as soybean meal is not known to be palatable to Tilapia. Similarly, Spirulina appears to reach a point when it becomes unpalatable to Tilapia and as a result, impairs feed intake and growth.

Similar and significant decreases in feed intake were observed when Spirulina content exceeded or was beneath 18.5% (6.5% fishmeal) in Red Tilapia diets (El-Sheekh et al. 2014). Olvera Novoa et al. (1998) observed feed intake decrease from 96.093 mg/day to 29.218 mg/day when increasing Spirulina from 20% to 42% of the diet. Additionally, they noted increased instances of feed rejection in diets containing high levels of Spirulina, which they related to the hardness of the Spirulina pellets. Spirulina hardens and takes on a crisp texture when extruded (Joshi et al. 2014) and feed texture is recognized as being important to fish feed acceptance (Adron and Mackie, 1978). We did not notice increased pellet rejection in Spirulina containing diets, but noticed rejections with SBM, suggesting that pellet texture may not have been an issue with increased Spirulina content over soybean meal. Hernandez et al. (2012) did not report any statistically significant changes in feed intake with changes in diet, nor were any noticeable trends observed. Hernandez et al. (2012) however, used Rainbow Trout, and palatability is species specific (Kasumyan and Døving, 2003). Given the available evidence, we would suggest that Spirulina inclusion in Tilapia diets is not limited by nutritional quality, but rather by palatability, and that Tilapia may be more sensitive to Spirulina inclusion than other species, such as Rainbow Trout.

Adding fishmeal to a diet containing 15% Spirulina did not significantly improve any growth performance measures. SP15 and FM2, containing 0 and 2% fishmeal, tended to outperform FM10 and CC, suggesting that fishmeal can be completely replaced in grow-out Nile Tilapia diets. Similarly, Teimouri et al. (2013) and Nandeesha et al. (2000) found that fishmeal can be entirely replaced by Spirulina in Rainbow Trout and Indian Carp respectively.

Both FM10 and CC exhibited lower feed intake values (1.86 % consumed/day and 1.68%/day) than SP15 and FM2 (both 2.17% consumed/day). Fish have typically evolved to depend on several different types of feed, which suggests that they grow better on, and ultimately prefer, diets based on multiple protein sources (Goda, 2007). While we found that SP15, a diet with two main protein sources, may have outperformed a solely soybean meal diet, we did not find that adding a third protein source resulted in improved growth or feed intake.

Our results suggest that Spirulina palatability is responsible for decreased growth performance with increased inclusion but further research is required. We ensured that all essential amino acids and vitamins and minerals were available in excess of what was required in Nile Tilapia diets but we did not monitor their uptake efficiency and therefore cannot know if they were sufficiently absorbed by the fish for metabolic utilization. While Sarker et al. (2016) suggest that Spirulina essential amino acid digestibility is above 90%, we did not specifically test for this, and are not able to make any claims about either essential amino acid or inorganic nutrient absorption. Additionally, we did not analyze physiological changes that may have occurred when using Spirulina, or when combining Spirulina, soybean meal, and fishmeal. Overturf et al. (2013) suggest that this is an area of study requiring more attention. It may have yielded additional complementary or contradictory results to support or challenge our palatability argument and we strongly recommend further research.

We included both male and female fish in grow-out trials, which may have influenced growth results. Often, female fish are excluded from experimental trials for fear that they will exhibit less than maximum growth, and will entice male fish to redistribute energy away from growth and towards reproductive development. While there were imbalances in the number of males and females between tanks, there was no imbalance between the feed treatments, and no relationship between weight gain and the distribution of males or females in a tank was observed (Appendix A4). We did, however, observe several instances of reproductive behaviour in almost every experimental tank, including increased territoriality, courting, leading, and copulation with mouth brooding. Although experimental tanks were checked for mouth brooders twice daily, and once discovered mouth brooders were unburdened from their eggs, the diversion of energy from growth to reproductive organs likely occurred. There is no evidence to suggest that any one feed promoted more reproductive behaviour than another, and we assume that all feed treatments were affected equally. That being said, instances of hyper-aggression, where one fish would dictate and limit the feeding behaviour of other individuals were especially apparent in tanks with higher male to female ratios. We suspect that these types of behaviors increased feed intake variation, and may have affected growth performance results.

Dietary protein content has been observed to increase nitrogenous waste output in Tilapia regardless of provenance (Brunty et al. 1997; Caulton 1978). We observed that the basal levels of TAN increased with increased feed protein content and that the experimental TAN levels did not. While CC had the highest protein content and the lowest overall experimental TAN levels, SBM had the lowest protein content and the second lowest experimental TAN. The inclusion of Spirulina appears to have had an effect on TAN but was not, however, the sole determinant as TAN levels decreased as Spirulina levels increased.

Feed intake and growth were the best predictors of TAN. Feed intake and growth are related and ultimately contribute to the same process resulting in the formation of ammonia. Increasing the amount of feed administered to a tank increases the absolute value of protein-nitrogen introduced. With increased nitrogen presence, there is an increased potential for ammonia formation because of the wealth of available amino acids (Brunty et al. 1997). Because waste production is the result of the same metabolic processes that lead to growth, increased growth will likely, unless all amino acids are present in the exact right proportions, lead to an increase in ammonia production.

While SP15 demonstrated strong growth, high feed intake values and high TAN levels, CC, which promoted more growth and had a similar feed intake, did not. The stability of each of the diets can account for the observed TAN levels in conjunction with its feed intake and growth results. Feed stability was not included in the regression model as it was directly correlated with feed type. Increased Spirulina content was correlated with decreased pellet stability and poor pellet stability results in nutrient leaching. Any increase in feed demand, which in our study is analogous to feed intake, results in more feed administered to a tank and therefore more opportunity for unstable feed pellets to shed nutrients and cause water fouling. While CC was one of the most demanded feeds and had a high protein content, it had significantly higher pellet stability and did not leach its available nutrients to the same extent as SP15.

Despite similar feed intake levels, identical Spirulina contents, and similar growth results, FM2 had significantly lower TAN than SP15. It is unlikely that FM2 contained less protein than SP15 (fishmeal contains more protein than soybean meal), and basal TAN levels for the two diets were identical. Pellet stability may have therefore played a role in decreasing TAN output in the fishmeal containing experimental diets. Hashim and Mat Saat (1992) suggested that lipid content has a positive correlation with pellet stability. Fishmeal tends to have higher lipid content than Spirulina (Olvera-Novoa et al. 1998; Sarker et al. 2016) and soybean meal, which suggests that our fishmeal containing experimental diets would have a higher lipid content than our non-fishmeal containing diets, and potentially, higher feed stability. Alternatively, the increased digestibility of a diet containing less soybean meal could have increased protein efficiency rates thereby decreasing TAN production. Fishmeal does not contain anti-nutritional factors that reduce digestibility and its protein digestibility ranges between 87-92% (Hanley 1987; Wantanabe et al. 1996; Maina et al. 2002) whereas soybean meal's is approximately 85% (Ogunji, 2004). Although the presence of Spirulina may have increased the digestibility of soybean meal (James et al. 2006), we are unsure to what extent, and as such, must consider compromised digestibility as a possible factor contributing to the observed decrease in total ammonia nitrogen with increased fishmeal content.

In contrast to total ammonia nitrogen, total phosphorus was largely accounted for by feed type. TP increased with decreased soybean meal and increased Spirulina content, and was highest for CC which mirrored the basal TP levels. Hernandez et al. (2012) observed a similar increase in metabolic phosphorus excretion with increased Spirulina content, and observed the highest TP outputs from a commercial control and a 60% fishmeal control. While we observed a trend towards higher TP in FM containing diets, FM2 and FM10, did not display significantly increased TP values over SP15. Diets containing fishmeal were observed to increase total phosphorus content of Rainbow Trout feces over diets containing soybean meal and Spirulina (Hernandez et al. 2012). Fecal phosphorus waste must be mineralized and solubilized by the aquatic environment to be accounted for in the TP testing performed in this study (Lim et al. 2008). Our experimental set up may have eliminated or reduced the contribution of fecal phosphorus degradation on TP results, thereby overlooking the effect fishmeal may have had on TP output. The results of this study suggest that increasing Spirulina levels increased the amount of digestible phosphorus in the diets past the level required to maximize growth and minimize phosphorus excretion (Lim et al. 2008).

The commercial control was found to be significantly more palatable than the other experimental diets during the self-feeder palatability trials. This result is not completely reflected in the grow-out experimental results, suggesting that while the palatability study may have been able to detect relative feed preferences, it was not predictive of growth.

Given these findings, short-term palatability testing may be better suited to pre-test candidate feeds before investing time and resources into a long term grow-out experiment. Fish can be slow to adopt a novel feed product, and long-term studies are required to decipher whether or not the delay in feed adoption will retard growth. The self-feeder method implemented in this study is not able to distinguish between an unpalatable diet which would lead to feed intake restriction and retarded growth, and an unpalatable diet that would be adopted over the time, ultimately not affecting growth.

This experiment also attempted to evaluate the use of enhancers to improve the palatability of a diet containing Spirulina. Mixtures of attractants are more effective than individual components and it was suspected that adding a well-known enhancer could further improve SP15 performance (Kubitza, 1995 scielo website). We did not, however, find that either citric acid or dried basil leaves had a significant effect on Tilapia feed demand. Given that the literature concerning citric acid's ability to increase feed intake is relatively well documented, we suspect that SP15 intake may have reached a peak. A true test of this, unfortunately, would have been to test the enhanced citric acid diet directly against CC, which significantly outperformed SP15 in previous self-feeder trials. A long-term evaluation of feed intake may have also yielded more conclusive results about the use of feed enhancers.

Despite the lack of significant improvements in feed palatability with feed enhancers observed in the present study, more research concerning the use of citric acid and dried basil leaves as enhancers should be undertaken. Feed preferences are species and life stage specific (Adams et al. 1988), and while Tilapia have found SP15 palatable, other species, or life stages of Tilapia, may not have (Kasumyan and Døving, 2003). The use of feed enhancers may therefore be necessary to increase the palatability of a Spirulina containing diet for another species, or another life stage. Additionally, both citric acid and dried basil leaves have both proven and suspected benefits that range beyond their taste enhancing abilities, which could warrant their inclusion even without feed intake benefits.

Ultimately, it would have been interesting to improve the palatabilities of our less palatable Spirulina diets. In performing this experiment we may have been able to determine whether feed intake, and not nutritional inadequacy, is limiting Spirulina inclusion past 15%. That being said, the profitability analysis suggested that a more judicious use of Spirulina is preferred and higher levels of Spirulina are therefore unlikely to be adopted by commercial enterprises.

The operation of the self-feeders used in any experiment should be questioned (Arande et al. 2000). Given the mechanism by which feed was distributed from our feeders, we had no method of testing the exact amount of feed released per unit effort or the uniformity amongst the feeders. We were however able to make several assurances of feeder function through the feed and feeder production methodology. First of all, the size ratio between the feed pellet and the release holes on the feeder is the ultimate factor determining the release of a pellet. Each of the experimental feeds was created using the same methods, and therefore we maintained the same feeder hole diameter:

feed diameter ratio. CC was not the same size as the experimental feeds so we designed larger holes with the same pellet diameter to hole diameter ratio. The ease with which any of the feeds were released from a self-feeder should have therefore been similar. Secondly, we rotated self-feeders between feeds to avoid any possibility that a particularly loose or difficult feeder was concentrated in one feed treatment. We do not assume that this methodology was flawless, but, given the measures we took to avoid feeder efficacy variation, we can assume that any error caused by the feeders was minimal and spread across feed treatments.

Given the cost of purchasing Spirulina, a shrewd use of the micro algae is recommended (Sarker et al. 2016). We evaluated four costs of Spirulina, and across all but the most expensive (the non-bulk price we paid for the Spirulina used in this experiment), diets containing 15% Spirulina were more profitable than diets containing only soybean meal. At every price, Spirulina was more expensive than soybean meal, and since growth results tended to relate to feed treatment, we have concluded that the cost of incorporating Spirulina at 15% is outweighed by the profits earned through fish flesh production. SP30 profitability also outperformed SBM when Spirulina was sourced at under 1000$/t but given the negligible differences between SP30 and SBM growth rates, we are less convinced by these results. When evaluating the fishmeal containing diets, which maintain 15% Spirulina inclusion and substitute inexpensive soybean meal for more expensive fishmeal, FM2 had a virtually identical profit index to SP15. This suggests that, while it may be financially feasible to use fishmeal in a Spirulina and soybean meal diet, it is not ultimately beneficial in terms of growth or profitability. The use of 10% fishmeal is even less profitable and leads us to conclude that the elimination of fishmeal from the diets of grow-out Nile Tilapia is profitable when replaced with soybean meal and 15% Spirulina.

Additional literature suggests that a fishmeal-free Spirulina containing diet can lead to other profit enhancing benefits that we did not specifically explore in this study. Not only can Spirulina inclusion potentially reduce fish mortality and increase immune activity, but a fish fed Spirulina instead of fishmeal can be marketed as an ecologically responsible food item. This allows the fish to fetch a higher market price and potentially offset the cost of using Spirulina (Habib et al. 2008). Giant River Prawn (*Macrobrachium rosenbergii*) farmers found that supplementing 5-20% Spirulina offset the initial cost of using Spirulina and provided a significant cost-performance ratio advantage (Nakagawa and Gomez-Diaz, 1975). That being said, sourcing an adequate amount of Spirulina at a profitable price may be difficult. Efficient in-house production, where possible, is recommended.

In conclusion, there was no significant difference in growth performance between our Spirulina containing diets, our fishmeal containing diets, and the commercial control. Observed trends and the profitability index would suggest that Spirulina inclusion be limited to 15% to avoid the negative effects that high levels of Spirulina may have on feed intake and therefore growth. Palatability results suggest that increased Spirulina inclusion leads to decreased feed demand, and that citric acid and dried basil leaves do not affect the palatability of a diet containing 15% Spirulina. Fishmeal inclusion in a Spirulina containing diet is not necessary for growth or palatability measures and should be avoided due to the increased costs associated with fishmeal inclusion over soybean meal. Spirulina inclusion appears to have an effect on both nitrogenous and phosphorous waste output, but total phosphorus output is lower than that of a commercial Tilapia diet, and it is likely that total ammonia nitrogen output can be remedied through improved manufacturing methods. Overall, we recommend that Spirulina and soybean meal be adopted to replace fishmeal in Nile Tilapia diets but further research is necessary. The effects of Spirulina on nutrient uptake and use should be clarified, and additional studies regarding the previously unexplored areas of palatability thresholds, the use of enhancers, waste production, and the overall cost of using Spirulina should be thoroughly investigated. While this study has advanced our understanding of Spirulina as a protein source in Tilapia diets, further research is required to diversify aquafeed ingredients in a way that promotes aquafeed sustainability and aquaculture growth.

Summary:

1. Spirulina was assessed as a potential protein source in grow-out Tilapia diets according to pellet stability, growth performance, palatability, waste production, and profitability.

2. Spirulina has an inverse relationship with pellet water stability. We suspect that Spirulina's high protein content compared to carbohydrate content may have negatively affected stability. Spirulina containing diets may require added binding agents, such as guar gum or carrageenan, in order to maintain high stability levels. It is unclear how much extrusion would have affected experimental diet stability.

3. All feeds resulted in weight and length gains and there was no significant difference in growth performance between feed treatments. The best model to predict Spirulina and fishmeal feed weight gain included percent feed intake and initial average weight of fish. These results suggest that the protein quality of Spirulina may not be the factor limiting high levels of inclusion in aquafeed. Instead, Spirulina appears to reach a point when it becomes unpalatable to Tilapia and as a result, impairs feed intake and growth. Given the available evidence, we would suggest that Spirulina inclusion in Tilapia diets is not limited by nutritional quality, but rather by palatability, and that Tilapia may be sensitive to Spirulina inclusion past 15%.

4. Basal levels of total ammonia nitrogen (TAN) and total phosphorus (TP) increased with increased Spirulina content but CC had the highest TAN and TP. Fishmeal inclusion did not affect TAN levels but increased TP.

4.1 Experimental total ammonia nitrogen (TAN) and total phosphorus (TP) levels are significantly affected by diet ($p<0.05$). In both Spirulina and fishmeal trials, tanks administered CC had significantly higher TP levels than the experimental diets, and significantly lower TAN than tanks administered SP15.

4.2 The inclusion of Spirulina appears to have had an effect on TAN but was not the sole determinant as TAN levels decreased as Spirulina levels increased. Feed intake and growth were the best predictors of TAN. Any increase in feed demand, which in our study is analogous to feed intake, results in more feed administered to a tank and therefore more opportunity for unstable feed pellets to shed nutrients and cause water fouling.

5. The commercial control (CC) was found to be significantly more palatable than the experimental Spirulina dies. No other significant differences in palatability were observed. Since there was no significant difference in feed intake or growth in the grow out experiment, we determined that the self-feeder method implemented in this study is not able to distinguish between an unpalatable diet which leads to feed intake restriction and retarded growth, and an unpalatable diet that is adopted over the time, ultimately not affecting growth.

5.1. Neither citric acid, an established feed enhancer, nor dried basil leaves were observed to positively impact the palatability of SP15. A long-term evaluation of feed intake may have yielded more conclusive results about the use of feed enhancers. Additionally, both citric acid and dried basil leaves have both proven and suspected benefits that range beyond their taste enhancing abilities, which could warrant their inclusion even without feed intake benefits.

6. The feed cost to produce one kilogram of fish (incidence cost) increased with increased Spirulina inclusion, increased fishmeal inclusion, and increased Spirulina price. The profit index for SP15 exceeded that of SBM across all Spirulina prices. The profit for SP30 only exceeded that of SBM with Spirulina process up to 1000$/t. SP45 did not outperform SBM at any price for Spirulina. While it may be financially feasible to use fishmeal in a Spirulina and soybean meal diet it is not ultimately beneficial in terms of growth or profitability.

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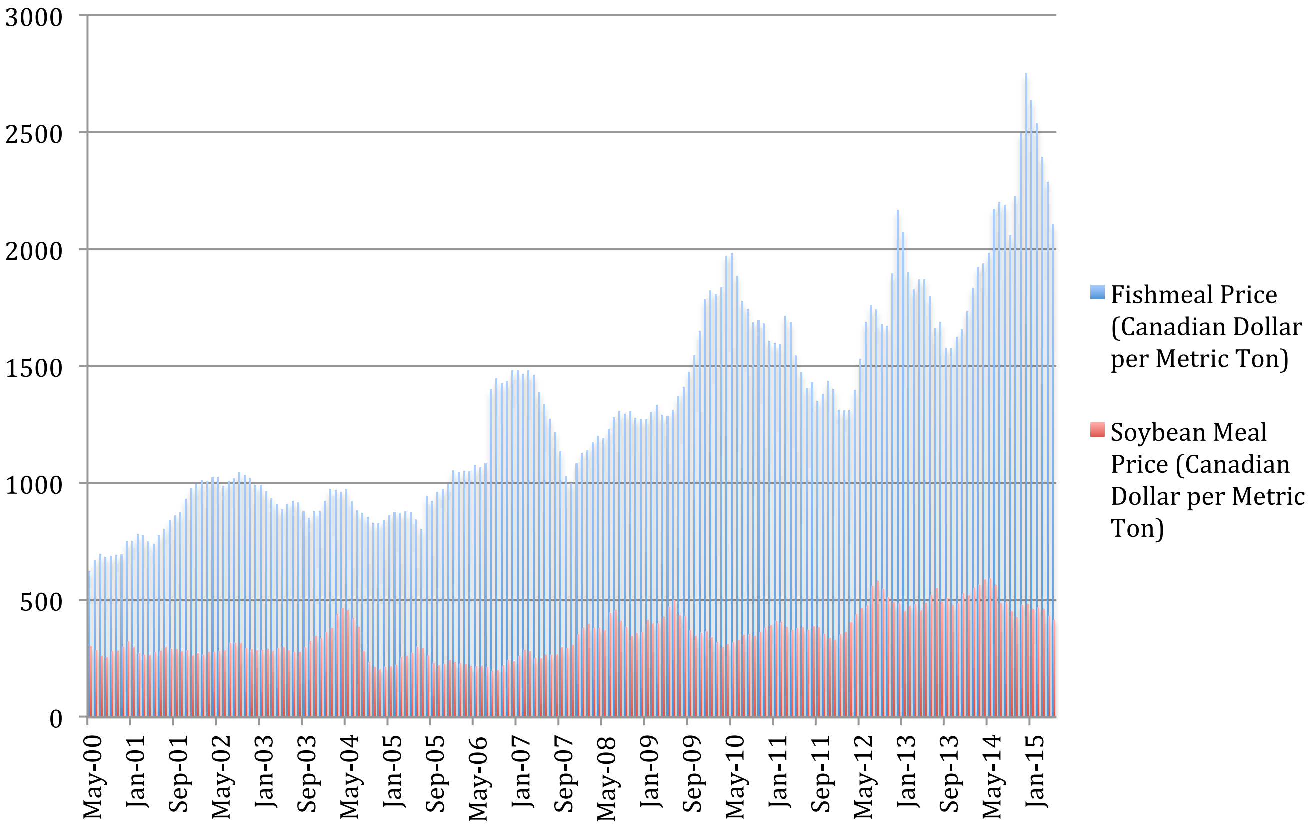
Appendix A1) Price fluctuations of fishmeal and soybean meal in Canadian dollars between May 2000 and June 2015.



Appendix A2) The mean growth and feed intake of tilapia fed Spirulina and soybean meal based experimental diets for 4 weeks by experimental block.

Block 1

Mean values	CC	SBM	SP15	SP30	SP45
Survival (%)	100	95	100	85	90
Initial body weight	82.1 (1.7)	82.6 (0.4)	81.5(0.7)	82.1 (0.9)	80.4 (0.3)
Final body weight	142.3 (6.3)	120.7 (10.4)	130.3 (9.4)	125.7 (18.4)	118.3 (4.0)
Weight gain (%)	73.3 (4.0)	46.1 (11.9)	59.8 (12.1)	53.0 (20.1)	47.2 (5.4)
Daily weight gain	2.15 (0.16)	1.36 (0.36)	1.74 (0.34)	1.56 (0.62)	1.36 (0.15)
SGR	1.96 (0.11)	1.34 (0.41)	1.66 (0.47)	1.49 (0.69)	1.38 (0.18)
Feed intake (% biomass/day)	2.32 (0.11)	2.22 (0.50)	2.49 (0.31)	2.36 (0.55)	2.33 (0.20)
FCR	0.99 (0.01)	1.77 (0.00)	1.11 (0.13)	1.49 (0.25)	1.52 (0.15)

Values are means (se) of 2-3 replicates.

Block 2

Mean values	CC	SBM	SP15	SP30	SP45
Survival (%)	100	93	85	90	86
Initial body weight	122.7	121.5 (2.1)	140.1 (16.2)	134.1 (13.3)	124.2 (0.8)
Final body weight	164.7	141.6 (4.9)	179.1 (26.3)	169.7 (18.1)	149.4 (3.43)
Weight gain (%)	34.2	16.5 (2.1)	25.6 (5.9)	24.8 (2.5)	20.3 (3.4)
Daily weight gain (%/day)	0.76	0.44 (0.10)	0.59 (0.36)	0.49 (0.17)	0.37 (0.15)
SGR	0.9	0.59 (0.14)	0.66 (0.39)	0.58 (0.16)	0.50 (0.14)
Feed intake (% biomass/day)	2.32	2.22 (0.35)	2.49 (0.42)	2.36 (0.30)	2.33 (0.37)
FCR	1.84	0.06 (2.45)	-0.08 (2.44)	0.92 (1.99)	3.53 (0.82)

Values are means (se) of 1-3 replicates.

Appendix A3) Total ammonia nitrogen and total phosphorus levels in tanks fed CC, SBM, SP15, SP30, and SP45 by block.

Block 1

	CC	SBM	SP15	SP30	SP45
Ammonia	2.45 (0.57)^{a}	2.62 (1.39)^{a}	5.61 (0.32)^{a}	4.60 (0.30)^{a}	3.24 (1.78)^{a}
Phosphorus	0.29 (0.07)^{a}	0.03 (0.03)^{b}	0.06 (0.00)^{b}	0.12 (0.00)^{b}	0.18 (0.00)^{ab}

Values in the same row with different subscripts are significantly different ($p< 0.05$). Values are means (se) of 2-3 replicates.

Block 2

	CC	SBM	SP15	SP30	SP45
Ammonia	0.57 (0.07)^{a}	2.50 (0.03)^{a}	3.01 (0.00)^{a}	1.91 (0.00)^{a}	0.92 (0.00)^{a}
Phosphorus	0.25 (0.00)^{a}	0.02 (0.82)^{b}	0.04 (1.39)^{b}	0.09 (1.53)^{b}	0.15 (0.09)^{ab}

Values in the same row with different subscripts are significantly different ($p< 0.05$). Values are means (se) of 1-3 replicates.

Appendix A4) Proportion of male fish in tank for fishmeal diet experiment as a predictor of weight gain (%) (n=3).