EVALUATION OF MICROALGAE AS A POTENTIAL FISHMEAL REPLACEMENT IN THE DIET OF OREOCHROMIS MOSSAMBICUS

A Dissertation

by

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This dissertation meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Algae biomass from strains isolated from Corpus Christi, TX water were evaluated as ingredients to replace fishmeal in diets fed to *Oreochromis mossambicus*. Strains were selected based upon ability to be cultured on inexpensive nutrient media, biochemical composition, and ability to achieve productivity of ≥ 0.10g/L. Further selection involved evaluation of test ingredients for apparent dry matter, protein, ingredient, and amino acid digestibility when fed to juvenile (~30g) tilapia. Digestibility diets consisted of 69% reference diet, 1% Cr_2O_3 as an inert marker, and 30% algal test ingredient. Apparent dry matter digestibility ranged from 50.7±0.02% to 70.6±0.07% in the *M. salina* and Spring mix 2014 (Spmix), respectively. The *Platymonas* sp. and the Spmix diets exhibited the highest dry matter digestibility (69.3±5.5% and 70.6±3.9%, respectively). The dry matter in the *Cylindrotheca* sp. (57.9±3.5%) and the *M. salina* (50.7±8.5%) diets were the least digestible. Results showed that there were no significant differences in regards protein digestibility in the formulated diets (n=3). Also, there were no significant differences between the digestibilities of the ingredients (ADCI). The *Platymonas* sp. and the Spmix had the highest ADCI 83.1 ± 30.0% and 85.3 ± 24.6%, respectively. Methionine was 93.0% digestible in *M. salina*, and lysine digestibility was highest in Spmix (88.5±0.46%).

In a subsequent 30-day feeding trial, ten ~0.170 mg tilapia were fed diets in which Spmix and *Platymonas* sp. (P) replaced various levels of fishmeal (0, 20, 40, 60, 80, 100%) in diets containing 40% crude protein. Weight gain (%), specific growth rate (SGR), final body weight, feed conversion ratio (FCR), protein efficiency ratio (PER), and percent survival were evaluated (n=5). Survival ranged from 96-100%. There were no significant differences in percent weight gain in the fish fed P20%-P80% diets. The FCR of the fish fed P20% and P40% were different

from those fed the P100% diet (P=0.0145). The PER of fish fed P20%, P40%, and P80% were also significantly different from that of the fish fed the P100% (P=0.0214) diet. Fish fed the P100% diet had the lowest FCR (1.19±0.14). All performance indices were similar for tilapia fed the Spmix diets. Results showed that both test ingredients could be used to replace fishmeal at high levels of dietary inclusion, 80% for *Platymonas* sp. and 100% for Spmix. This indicates high potential for replacement of fishmeal in tilapia feeds with marine microalgae.

DEDICATION

Dearest Logan,

I wrote this for you. I didn't realize when I began that our lives could possibly change so much in only a few years. I didn't realize that this was not about me following a childhood dream, it was about us working together to become stronger individuals and family. On my most tired, and my worst of days, thinking of your future pushed me through. Without you, none of this would have ever been accomplished.

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“Come to the edge," he said.
"We can't, we're afraid!" they responded.
"Come to the edge," he said.
"We can't, We will fall!" they responded.
"Come to the edge," he said.
And so they came.
And he pushed them.
And they flew.” - Apollinaire

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CHAPTER I: INTRODUCTION – GENERAL OVERVIEW OF PROTEIN AND AMINO ACID METABOLISM OF FISHES

The human population is estimated to exceed 9 billion people by 2050 (FAO, 2015). Providing food to meet the needs of an additional 2.5 billion people in 32 years will require both intensification of production and identification of non-traditional sources of nutrition, especially protein. One means of meeting this demand is through aquaculture and developing nutrient replete feeds allowing for production expansion. As such, the provision of compounded feeds represents the principal operational cost component in intensive aquaculture production (El-Sayed, 2006a). For most commercially cultured species, fishmeal has traditionally been the major source of protein and essential amino acids (Ng and Romano, 2013). Albeit a mainstay of aquaculture feeds, fishmeal has high market price volatility, its harvest negatively impacts marine food webs (Olsen and Hasan, 2012; Tacon and Metian, 2009), and is expensive (~$1,500/MT; Index Mundi, 2019). The price of fishmeal is expected to increase by 90% by 2024 due to increasing global demand from aquaculture (FAO, 2018). For these reasons, the evaluation of several potential alternative protein sources such as soybean, barley, rice, peas, canola, lupine, wheat gluten, corn gluten, yeast, other microbial biomass, insects, algae, and rendered animal products has occurred (NRC, 2011; Patterson and Gatlin, 2013; Salze et al., 2010; Schneider et al., 2004). Ultimately, the value of alternative feedstuffs to aquaculture is determined by how well new ingredients help meet the nutritional requirements of the animal and availability to feed manufacturers. Compounding the issue, many alternative ingredients could potentially compete with food security for humans either directly or indirectly by their inclusion in livestock and poultry feeds or by direct consumption (Tacon, 2015). Identifying "non-competitive" sources of protein requires extensive analysis to determine nutritional adequacy and extent of need (Salze et al., 2010; Zhou et al., 2005).

Overall, the majority of the cost of aquaculture feeds is associated with the protein sources. Therefore, it is crucial to reduce dietary inclusion level of fishmeal for the greater majority of aquaculture feeds, The use of costly ingredients (e.g., fishmeal) or those requiring extensive processing can cause aquaculture feeds to greatly exceed cost of typical animal feeds (Furuya and Furuya, 2010). Changes in market price of fish meal on the global market is highly unpredictable due to variations in supply resulting from perturbation in regional climate (e.g., El Niño vs. La Niña conditions) and demand.

Protein function

Protein is a macronutrient which is needed for animal cellular growth and development and often selectively used by aquatic organisms to partially meet energy requirements (Gonçalves et al., 2015). These high molecular weight molecules are incorporated into every cell in the body and serve significant functions in muscles, bones, organs, tendons, and ligaments. Proteins are vital for the maintenance of body tissues, including development and repair. They function in anabolism of nitrogenous compounds such as purines, polyamines, methylated compounds, thyroid hormones, creatinine, histamine, and taurine (Cowey, 1994). The carbon backbone of amino acids can be used in the tricarboxylic acid cycle (TCA) to produce adenosine triphosphate (ATP) or converted into glycogen or fatty acids (Cowey and Walton, 1988). Additionally, proteins assist in the synthesis of hormones such as insulin, secretin, facilitate molecular transport, and form antibodies to identify and destroy antigens such as bacteria and viruses, and are the primary constituent of enzymes (Buxbaum 2007). Therefore, protein intake is essential for rapid growth in young, growing animals (Santiago and Lowell, 1988).

Protein Quality

The amino acid composition of proteins and the degree to which amino acids are available to meet the metabolic needs of a given animal determine the nutritional quality of proteins. However, the quality of an aquaculture diet is not based purely on its overall biochemical composition. Aquaculture feeds are typically composed of several dietary ingredients and often contain a variety of proteins of dissimilar nutritive value, ultimately affecting biological availability of amino acids (Figueiredo-Silva et al., 2015). Numerous other factors contribute to the ability of the animals to digest and absorb nutrients, such as proteins. Feeding habits, developmental stage of the species, environmental temperature (Refstie et al., 2006), physiological status, physiological condition, and nutritional requirements of the animal (Santos et al., 2013) are all factors that contribute to nutrient utilization. Protein quality is largely determined by "digestibility," which refers to the measure of utilization of a nutrient group, or groups of nutrients. These dietary compounds are catabolized or passed through the digestive system via numerous pathways in the digestive system. The overall digestibility of feeds, dietary ingredients and nutrients is largely determined by the proportion of those constituents ingested versus excreted as feces or other metabolic products. The most commonly reported is "apparent digestibility" which is often determined indirectly by comparing the concentration of a nutrient in feed and feces to that of an inert biological marker, such as chromic or yttrium oxide (Austreng, 1978).

Other factors affecting nutrient and ingredient digestibility

The term, "digestibility" refers to the measure of utilization of a nutrient group, or groups of nutrients, as dietary compounds are broken down and absorbed by a diverse series of mechanisms in the digestive system. The overall digestibility of feeds, dietary ingredients and

nutrients is largely determined by the proportion of those constituents ingested vs. excreted as feces or other metabolic products. Apparent protein digestibility of feedstuffs can vary due to weather conditions, harvesting, storage, manufacturing, and milling. It can also be influenced by nutrient concentrations within a single component (Knapka, 1983). Processing of ingredients before, or during, the preparation of feeds also affects digestibility. For example, in proteins such as those in soybean meal, heat treatment is necessary and increases digestibility by inactivating anti-nutritional factors such as phytates and saponins (Francis et al., 2001). However, in some cases, excessive heat treatment can reduce protein digestibility by altering the solubility of the protein (Khan et al., 2003). These factors may also affect bioavailability of nutrients through changes in the chemical structure that could result in non-useful binding of nutrients with non-nutritive components (such as tannins) and reduce nutrient availability.

Ingredient digestibility is influenced by chemical and physical factors such as: particle size, chemical composition of ingredients, method of processing, and level of ingredient refinement. Biological factors such as feed intake, environmental factors, stress, age, and interactions with other dietary constituents (Khan et al., 2003) also effect digestiblity. Large variations in digestibility have been shown in respect to fishmeal in the diet of *O. niloticus*. Numerous studies have shown that the digestibility of protein of white fish meal in the diet of *O. niloticus* to be approximately 93% (Guimaraes et al., 2008; NRC, 2010; Watanabe et al., 1996). However, a digestibility value of 87% also was reported for fishmeal made from fish collected from different locations (Hanley, 1987). Additionally, the evaluation of ingredients for digestibility can be difficult in tilapia which have been observed “chewing” their food. They are known to repeatedly spit out formulated diets and ingest selected portions (Maina et al., 2002). Therefore, when

evaluating digestibility trial results, it is critical to specify the environmental conditions during the trial as well as the methods of ingredient collection, processing, and diet preparation.

Digestion of protein and amino acids

Gut retention time is a valuable metric to measure the potential for nutrient assimilation. Factors affecting rate of passage in fishes include fish health, size, life stage (Buddington and Doroshov, 1986; Torrissen and Torrissen, 1984), gut length (Bakke et al., 2010), and volume of feed (Hofer and Schiemer, 1981). Extrinsic factors such as water temperature (Hurst, 2004; Singh-Renton and Bromley, 1996), salinity (Day et al., 2011; Tsuzuki et al., 2007), size of meal (Andersen, 1999), and physical composition of feed also may affect digestion. For example, Horn and Messer (1992) showed that fish consuming poorly digestible feed are more likely to be continuous feeders resulting in reduced gut retention time (Uscanga et al., 2010). Fortunately, the role of enzymes in nutritional physiology and biochemistry of aquacultured species is well understood (Santos et al., 2013; Tengjaroenkul et al., 2000). Enzyme constituents of the digestive tract affect the ability of animals to digest and assimilate nutrients (Tengjaroenkul et al., 2000) in fish such as salmon (Storebakken et al., 1999), cod (Hjelmeland et al., 1983), and whiting (Anderson, 2001; Anderson, 1999). Variation in diet due to change in level and type of natural productivity, can alter the expression of the activity of digestive enzymes resulting in tilapia achieving a higher protein efficiency ratio (PER) when raised in ponds.

Amino acid requirements of tilapia

Dietary proteins for aquaculture species are composed of amino acids (AA) derived from a variety of ingredients and, as such, require substantial evaluation prior to use commercial diets.

A large number of AAs have been identified, but only 20 are commonly observed in nature. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are considered essential in many species of fish and crustacean (Wilson and Halver, 1986). It has since been suggested that tilapia also have a requirement for these ten essential amino acids (EAA) (Jauncey, 1998; El-Sayed, 2006a; Santiago and Lovell, 1988; Wilson and Halver, 1986). Amino acid requirements can be met by a variety of protein-based ingredients. Based on biochemical composition it appears as though a wide variety of sources have potential for providing EAAs in fish feeds. The extent to which the requirement is met depends upon quality (digestibility) of the protein or on the amount of feed the animal consumes. In general, as protein quality decreases, the amount of protein included in the diet must increase.

The overall amount of AAs that are bioavailable to the organism determines the nutritional quality of a particular protein source. Therefore, dietary proteins should also contain appropriate levels of EAAs. At a basic level, animals do not have a protein requirement they have an AA requirement.

The EAA requirements of tilapia, *Oreochromis niloticus* (Nile tilapia) have been the most extensively researched; whereas relatively few studies have addressed this topic for *Oreochromis aureus* (blue tilapia) and *Oreochromis mossambicus* (NRC, 2011; Jauncey et al., 1982; Wilson, 2002). The optimum dietary level of EAAs for tilapia is variable and dependent on, weight, density, biotic and abiotic factors present in the environment, (El-Sayed, 2006b; Jauncey, 1998) or the source of feed ingredients in a formulated diet (NRC, 2011). Additionally, EAA requirements change at different life stages, and to a certain extent, by species of tilapia. Jauncey and Ross, 1982 estimated the protein requirement of fingerling and fry *O. mossambicus* to range between 40-50% of the diet. Jauncey (1982) also quanified the EAAs required by juvenile *O.*

mossambicus (% of diet) were; arginine (Arg) (1.13), histidine (His) (0.42), isoleucine (Iso) (0.80), leucine (Leu) (1.35), lysine (Lys) (1.51), methionine (Met) (0.40), phenylalanine (Phe) (1.00), threonine (Thr) (1.17), tryptophan (Try) (0.17), and valine (Val) (0.88) Similar requirements have been identified for juvenile *O. niloticus*. Santiago and Lovell (1988) quantified EAA requirements (% of diet) in *O. niloticus* which have been estimated to be; Arg (1.18), His (0.48), Iso (0.87), Leu (0.95), Lys (1.43), Met (0.75), Phe (1.05), Thr (1.05), Try (0.28), and Val (0.78). Furuya et al. (2006) estimated requirement of lysine in the diet of adult *O. niloticus* to be (1.04-1.74). Recently, two additional studies identified His and Lys requirements of juvenile *O. niloticus* as 0.31 and 0.58 of crude protein, respectively (Michelato et al., 2016, 2017).

Functions of essential amino acids in tilapia

Bioavailability of first-limiting EAAs typically determines fish growth and nitrogen retention (Furuya and Furuya, 2010). In omnivorous fish such as tilapia, alternatives to fishmeal are frequently used to supply protein, some of which are deficient in EAA. In most cases, contained intensive aquaculture (i.e. lined ponds or tanks) does not provide the opportunity for the animal to "select" a diet that meets nutritional requirements. Therefore, nutritional requirements are met by inputs chosen by the farmer, either as feed or by manipulation of pond natural productivity. Amino acid deficiencies may cause anatomical abnormalities such as cataracts (Cowey et al., 1992; Poston et al., 1977), reduced growth (Poston et al., 1977), reduced feed conversion efficiency (NRC, 2011), fin erosion (Walton et al., 1984), scoliosis, lordosis (Walton et al., 1984), mortality (Poston et al., 1977), and other maladies.

The rising cost of fishmeal has led to increased frequency of using non-fishmeal-based proteins such as yeast, meat meal, bone meal, blood meal, hydrolyzed feather meal (FAO, 2018),

and plant-based proteins such as soybean, oat, barley, rice, canola, and wheat gluten in aquaculture feeds. Unfortunately, many of these ingredients are deficient in methionine (FAO, 2018) and lysine (El-Sayed, 2006; FAO, 2018; Michelato et al., 2016). Inadequate intake of methionine is shown to reduce growth, feed efficiency (Belghit et al., 2014; Figueiredo-Silva et al., 2015; Furuya et al., 2013; Tulli et al., 2010), and can increase mortality (Goff and Gatlin, 2004). It has been estimated that tilapia require ~3.2% of their protein to contain methionine and that it was typically the most-limiting EAA (Cooper, 1986). Later it was discovered that cysteine can be synthesized from methionine. Cysteine has been shown to spare dietary methionine requirements by 40 to 60% in fish (Goff and Gatlin, 2004; Khan and Abidi, 2011; Nguyen and Davis, 2009). However, this conversion is irreversible; therefore, methionine is considered essential, regardless of cysteine availability. The ability to convert one sulfur-containing amino acid to another results in the use of total sulfur amino acid (TSAA) requirement as another index of ingredient quality. Thus, TSAA requirements can be met in most animals by the use of an appropriate mixture of methionine and cysteine.

A major role of lysine is to regulate carnitine synthesis in the liver and skeletal muscle (Farhat and Khan, 2014). Carnitine assists in transportation of long chain fatty acids into mitochondria for β-oxidation, reducing the fat proportion in the body (Berge et al., 1998). Additionally, lysine is a structural component of collagen and it blocks enzymes that break down collagen and also assists in maintenance of osmotic pressure and pH balance in body fluids (Chiu et al., 1988). It is associated with nitrogen retention (Cao et al., 2012), increases in size and length of muscle tissue via hyperplasia and hypertrophy (Michelato et al., 2016; Valente et al., 2013), and enhances protein deposition in the body (Furuya and Furuya, 2010; Hamid et al., 2016). Lysine supplementation in deficient diets for numerous fish species results in higher overall growth (Zhou

et al., 2007), increased fecundity (Hamid et al., 2016; Lahnsteiner, 2010), and greater immunity (Zhou et al., 2009).

Finding alternative feed ingredients in *Oreochromis* spp. diets to supply Met and Lys is important as these EAAs are notably deficient in many plant protein ingredients (e.g., oilseed meals) (FAO, 2018), and is often a limiting EAA in fish feeds (Gatlin et al., 2007; Sarker et al., 2018). Plant products are frequently used in aquaculture diets to replace fishmeal as a protein source as they are significantly less expensive (Indexmundi.com, 2019). Therefore, certain AAs must be supplemented in these diets to meet requirements for growth and survival.

Tilapia as an aquacultured species

One common aquacultured species with generally lower protein requirements than its carnivorous counterparts is Mozambique tilapia (*Oreochromis mossambicus*). This member of F. Cichlidae is native to tropical and subtropical Africa (Nico et al., 2018). Some members of *Oreochromis* spp. are euryhaline and are reported to survive in salinities ranging from 0 to 120 ppt (Trewavas, 1983). Tilapia are known to have a rapid growth rate, wide range of salinity tolerance, high fecundity, and swift acceptance of artificial feed (El-Sayed, 2006b). Additionally, they are also known to have high disease resistance, tolerance to crowding (Ng and Romano, 2013), ease of adaptation to enclosed waters (He et al., 2013), and reduced fishmeal content requirement in comparison to other aquacultured species, such as salmonids (Stickney, 1997). These properties are among the reasons *Oreochromis* spp. are the second-most commonly farmed fish worldwide (Ng and Romano, 2013). Studies on *Oreochromis* sp. conducted in the 1960s and 1970s suggest that the diets of some species reflect change over time in relation to variations in their natural habitat, such as eutrophication (Zengeya and Marshall, 2007) or season (Maitipe and DeSilva,

1985). However, all known tilapia species select varying food sources at different life stages and sizes (Turker et al., 2003; Zengeya and Marshall, 2007). These numerous properties make them an ideal candidate for aquaculture. Additionally, the potential for lowering fishmeal content of production diets for tilapia has been demonstrated (Botaro et al., 2007) and the issue of dietary protein content in feeds for the genus *Oreochromis* spp. at different life stages has been extensively researched (Table 1).

Table 1. Estimated requirement (%) of protein in the diet of *Oreochromis* spp.

Species	Lifestage	Est. Requirement (% Diet)
O. niloticus	Fry/Broodstock[1,2,3]	35-45
	Fingerlings[4,5,6]	30-40
	Adults[1,3,5]	26-30
O. mossambicus	Fry[7]	40-50
	Fingerlings[8]	40
	Adults[7]	30-35
O. aureus	Fingerlings[9]	56
	Adults[9]	34

[1] Al-Hafedh, 1999
[2] El-Sayed and Teshima, 1992
[3] Siddiqui et al., 1998
[4] Abdelghany, 2000
[5] NRC, 2011
[6] Wang et al., 1985
[7] Jauncey and Ross, 1982
[8] Jauncey, 1982
[9] Winfree and Stickney, 1981

Use of algae in tilapia diets

Tilapia are omnivorous fish, capable of utilizing benthic and planktonic algae as nutrient sources, making them an excellent model organism for evaluating alternative plant sources for fishmeal replacement (Furuya et al., 2001; Hanley, 1987; Koch et al., 2016; Köprücü and Özdemir, 2005; Moxley et al., 2014; Sklan et al., 2004; Vidal et al., 2017). The primary focus of research to date has been on freshwater algal species that are novel, non-commercial, and produced in limited supply (Ng and Romano, 2013). Certain species of algae can produce from 30 to 300 times more oil per ha of land used (Ziolkowska and Simon, 2014). Whereas land crops may take months to grow for harvest, many species of microalgae have doubling times on the order of hours. As opposed to traditional crops, microalgae are capable of achieving much higher productivity, relatively high lipid content, and protein content (Branco-Vieira et al., 2017; FAO, 2016) compared to corn, soybeans, and palm. Additionally, marine species of microalgae also require minimal use of domestic water in comparison to fresh water species. Therefore, the species of marine microalgae capable of being commercially mass-produced warrant evaluation for use in aquaculture feeds.

To accomplish this, it is necessary to first evaluate and characterize traditional, albeit unsustainable, nutrient sources used in feeds. As the principal ingredient in aquaculture feed, fishmeal contains a high (~65% dry matter) level of protein, is replete in essential marine fatty acids, and provides substantial levels of vitamin E, minerals, and phospholipids. It also possesses potent chemoattractive qualities suitable for aquaculture feeds (NRC, 2011). Therefore, to provide dietary protein traditionally derived from high-protein ingredients such as fishmeal, the protein in alternative ingredients and sources must be both nutritionally and economically comparable. Researchers have been attracted to microalgae and macroalgae for use as a food supplement due

to their interesting physical and chemical composition (Bennamoun et al., 2015). However, the chemical composition of microalgae, including the amino acids and fatty acids, is extremely variable (Zhukova and Aizdaicher, 1995). The differences in chemical content are affected by numerous variables such as species, salinity (Khatoon et al., 2014), temperature, and light intensity (Morris et al., 1974). They are also affected by nutrient availability (Sharma et al., 2012), environmental conditions, geographic area, season, life stage, (Mabeau and Fleurence, 1993) as well as manufacturing and drying processes. Also, many algal species are a rich source of nitrogenous compounds, especially proteins and amino acids (Campanella et al., 1999).

Algal biomass is not currently viewed as competitive with human food resources and has commercial applications such as food colorants, dyes, cosmetics, pharmaceuticals, pollution control, and food additives (Mata et al., 2010). Additionally, algal biomass can serve as a value-added co-product (Suganya et al., 2016) that could potentially stimulate the growth of the biofuel industry. Due to all the aforementioned uses, aquatic plant production has increased 44% since 2012, with 30.1 million tonnes provided by aquaculture in 2016 (FAO, 2018). Commercialization of marine microalgae may have further advantages over freshwater algae as it does not directly compete with water demand for domestic human consumption. Furthermore, many species of marine microalgae can provide substantial ecosystem services to humans such as carbon sequestration or reduced nitrogen and phosphorous output into the environment.

Published research on the use of algal species as an aquafeed is increasingly common. Previous studies have shown that incorporation of algal biomass into aquaculture feed can replace up to 50% of the fishmeal component in the diets of various species of freshwater fish (Badwy et al., 2008) and 10% in saltwater species such as *Sciaenops ocellatus* (Patterson and Gatlin, 2013) without affecting fish performance. *Nannochloropsis salina* meal used as a crude protein source

in tilapia fingerling (~12g) diets is capable of replacing soybean meal by up to 100% (Gabadamosi and Lupatsch, 2018). Additionally, algae consumption has also been found to lower fish susceptibility to illness or stress, and decrease mortality (Henson, 1990; Mustafa and Nakagawa, 1995).

Objectives

The primary objectives of this study are 1) to identify local algal strains and mixes of strains as potential candidates for fishmeal replacement based upon biochemical composition and productivity; 2) to further select algal ingredients by determining dry matter and protein digestibility by juvenile *Oreochromis mossambicus*; and 3) to determine maximum level of dietary replacement of fishmeal by these ingredients via growth trials with tilapia.

CHAPTER II: BIOCHEMICAL AND BIOMASS PRODUCTIVITY EVALUATION OF MARINE MICROALGAE AS A POTENTIAL FISHMEAL REPLACEMENT IN OREOCHROMIS MOSSAMBICUS (MOZAMBIQUE TILAPIA) DIETS.

Abstract

The primary objectives of this study were to 1) screen algal isolates as candidates for high-density outdoor culture; and 2) to identify from those species, or species mixes, candidates with appropriate nutrient profiles for use in fishmeal replacement diets for *Oreochromis mossambicus*.

A total of 24 species were analyzed for productivity rate in inexpensive media consisting of a nutrient blend (2.0 mM nitrogen (N) from ammonium sulfate, 0.13 mM phosphorus (P) from pH balanced phosphoric acid, and 0.07 mM iron from iron sulfate) at a 16:1 N:P ratio. The resulting biomass was used to inoculate 557-L outdoor raceways for May and October trials. There was no significant difference for growth rate within seasons for all surviving candidates. The maximum productivity of the individual summer cultures was; *M. salina* (49.43 ± 23.59), *Cylindrotheca* sp. (40.18 ± 20.45), and *P. tricornutum* (7.83 ± 12.10), and their mixture reached (42.29 ± 20.77 g/afdw/m^2/day). The fall cultures had lower maximum productivity rates relative to the summer 2016 cultures. All algae analyzed had a crude protein content higher than that of traditional soybean meal. Cyanobacteria and *M. salina* biomass contained a crude protein content comparable to fishmeal (64.5% and 62.17%, respectively). Crude lipid was 18.58% in *M. salina*, and *Platymonas* sp. contained the least (10.05%). The cyanobacteria used in this study contained higher methionine levels in comparison to other algal cultures (1.35%) but none had the EAA content of either fishmeal or soybean meal. However, all cultures contained an essential amino profile conducive for use in an aquaculture feed formulated for *O. mossambicus.*

1. Introduction

Tilapia are omnivorous fish, having a variable diet of both benthic and planktonic algae. This dietary plasticity makes them an excellent organism to use in the evaluation of alternative plant feedstuffs as potential replacements for fishmeal (Furuya et al., 2001; Hanley, 1987; Koch et al., 2016; Köprücü and Özdemir, 2005; Moxley et al., 2014; Sklan et al., 2004; Vidal et al., 2017). Different feeding behaviors and dietary selections have been found among species of the same genus (Zengeya and Marshall, 2007) and species (e.g. *Oreochromis mossambicus*) (Maitipe and DeSilva, 1985) of *Oreochromis*. Studies suggest that the diets of some species of tilapia change over time due to alterations in their natural habitat, eutrophication (Zengeya and Marshall, 2007), or availability of food sources that are dependent on certain climates (Maitipe and DeSilva, 1985). Other studies show that the feeding habits of tilapia may not be affected by seasonality (Hodgkiss and Man, 1977). All known species of chichlid select varying food sources at different life stages and sizes (Turker et al., 2003; Zengeya and Marshall, 2007) and a large portion of their natural diet throughout all life stages consists of green algae (Getachew and Fernando, 1989; Spataru and Zorn, 1978; Zengeya and Marshall, 2007). *Oreochromis mossambicus* have been shown to be primarily phytoplanktivorous in certain regions (Maitipe and DeSilve, 1985). For this reason, several fishmeal replacement studies with tilapia have been performed using rapidly growing species of algae (Gbadamosi and Lupatsch., 2018; Mahruzur et al., 2018; Teuling et al., 2017; Teuling et al., 2019).

Few studies have evaluated the use of marine microalgae in tilapia diets. Microalgae has the potential to replace or reduce dependence of the aquaculture industry on fishmeal by having a positive effect on protein and lipid deposition in muscle, reduction of nitrogen excretion into environmental receiving streams, improvement of disease resistance, increase in n-3 fatty acid

profile in the muscle tissue, and carcass quality (Becker, 2004). Becker et al., (2007) evaluated 40 species of microalgae from seven classes and found that all the species examined had similar amino acid composition. Studies have shown that incorporation of algal biomass into aquaculture feed can replace up to 50% of fishmeal in the diets of various species of freshwater fish (Badwy et al., 2008). However, until recently research has primarily focused on microalgae as a minor ingredient due to high cost of algae production, and lack of nutrient availability due to the cell walls in some species. As technologies have advanced, studies have begun evaluating algae as a more prominent ingredient (Mahruzur et al., 2018).

For these reasons, the present study chose to look at multiple species, and mixes of species, to produce biomass for this as well as the following trials (Chapter III and IV) for potential fishmeal replacement. These species/genera were; *Microchloropsis salina, Phaeodactylum tricournutum, Platymonas* sp., *Cylindrotheca* sp. and an unknown filamentous cyanobacterium. Also included in the study were three mixes consisting of 1.*) M. salina, P. tricornutum,* and *Amphora* sp.; 2.) *M. salina, Platymonas sp.*, and *P. tricornutum*; and 3.) *M. salina, P. tricornutum,* and *Cylindrotheca* sp.

Microchloropsis salina (CCMP 1776), previously known as *Nannochloropsis salina*, is a member of class Eustigmatophyceae characterized as a yellow-green unicellular alga. *M. salina* is halotolerant, performs well in a wide range of temperatures, and contains chlorophyll *a*. This alga is capable of producing high biomass production numbers and can contain a cellular lipid content of >30% (Huysman et al., 2015; Ma et al., 2014). These characteristics have resulted in this species becoming a model organism for biofuel production.

Platymonas is a genus of the chlorophyte Family Volvocaceae. The strain of *Platymonas* used in the present research was isolated from local waters and was not speciated. This organism

was chosen due to the fact that many species of *Platymonas* are halotolerant and can survive salinities ranging from 20-200% seawater with no effect on growth rate (Hellebust 1976). Additionally, *Platymonas helogolandica* is a commonly used species in larval marine hatcheries in Asia. It is known to improve water quality and stabilize pH by utilizing excess nitrogen and stabilizing CO_2 concentration (Ge et al., 2016a). In a study in which this species was fed to *L. vannamei* larvae, the mean final weight, weight gain and FCR were significantly improved vs. animals fed a diet formulated to contain similar nutrient content as the treatment but with no *P. helogolandica* (Ge et al., 2016b). When larvae were challenged with *Vibrio parahaemolyticus*, those fed the highest amount of *P. helogolandica* had a survival rate of 91.1% vs 43.3% for the control.

Cyanobacteria are unicellular bacteria that are characterized by a blue-green hue. They can survive under a wide range of temperatures and salinities and perform well in environments with high irradiance and abundant nutrients. Many cyanobacteria are easily cultured (Castenholz, 1988) and are likely to contain a high level of protein in the appropriate environment (Dong et al., 2012). Cyanobacteria have also been shown to improve saturated fatty acids, and collagen content in fish (Liang et al., 2015). *Arthrospira platensis* (*Spirulina*) has been shown to enhance growth performance of tilapia when up to 30% of the diet was replaced (Velasquez et al., 2016). Additonally, they may also offer tissue protection and serve as an antioxidant in *O. niloticus* (Ibrahema and Ibrahim, 2014). Unfortunately, the use of cyanobacteria in aquaculture diets has also been shown to result in toxic microcystin accumulation in the muscle tissues of C*arassius auratus* (Liang et al., 2015), *Cyprinus carpio* (Li et al., 2014), and *Oreochromis niloticus* (Palikova et al., 2011).

In this study, diatomaceous algae were also evaluated. *Phaeodactylum tricornutum* is a member of class Bacillariophyceae. They are a diverse group that contain a frustule composed of silica (Lee, 2008). The biochemical composition of *P. tricornutum* with respect to essential amino acid and fatty acid content indicates high potential for use in production diets for tilapia (NRC, 2011; Zhukova and Aizdaicher, 1995) and amino acid profiles support its role as an ingredient in tilapia diets (NRC, 2011), luxuriant growth in cooler weather, and ability to maintain a high level of productivity under various environmental conditions when co-cultured with *M. salina* (Huysman et al., 2015). *P. tricornutum* has also exhibited high productivity when cultured in outdoor ponds (Goldman et al., 1975; Leviatan et al., 2014).

High rates of productivity are exhibited by many species of marine microalgae, and they have the ability to provide substantial ecosystem services to humans, most notably nutrient cycling and carbon dioxide sequestration. Therefore, algal biomass can provide value-added co-products (Suganya et al., 2016) that could potentially improve economics of the biofuel industry. Commercialization of marine microalgae may have further advantages in that its use of brackish or marine waters for culture as it does not directly compete with water demand for domestic human consumption.

2. Objectives

The primary objectives of this study were to 1) to isolate and screen local algae as candidates for outdoor algae biomass culture; and 2) to evaluate these strains for level of biomass productivity and biochemical composition under varied seasonal conditions as candidates for use in replacement of fishmeal in tilapia production diets.

3. Methods

3.1 Sample collection and methods

Water samples were collected in 500-mL plastic Nalgene (Nalgene Nunc International Corporation, Rochester, NY, USA) bottles in the vicinity of Corpus Christi, TX (Table 2) near the shoreline at the surface and at ~ 1m of depth. Samples were collected from six locations between 11 am and 3 pm in November 2015 and April 2016 to obtain a representative sample of regional algae from both winter and spring seasons. Locations were chosen to yield potential diverse algal species due to differences in salinity, level of nutrient input, and temperature. Algae were isolated by single cell and enriched with media for grow out.

Table 2. Algal Collection Sites December 2015 and April 2016.

Site	Location	Latitude	Longitude
Nueces Bay	Carbon Plant Road	27°49'32.12"N	97°28'35.41"W
Corpus Christi Bay	Indian Point Park	27°51'7.13"N	97°21'22.65"W
Redfish Bay	Aransas Pass	27°52'47.24"N	97° 6'7.92"W
Packery Channel	Packery Channel Park	27°37'38.24"N	97°13'2.02"W
Laguna Madre	Barney M. Davis Energy Center	27°36'27.45"N	97°17'53.11"W
Oso Bay	South Padre Island Drive	27°40'44.90"N	97°18'35.00"W

3.2 Criteria used for selection of algal species

Selection of algal species for subsequent biomass culture was based on the ability to be cultured under the following criteria: 1) salinity ≥ 28 ppt; 2) temperature tolerance typical of the average south Texas climate (17.1^{o}C – 27.5^{o}C); 3) dry matter protein content ≥ 40% in harvested biomass; 4) ability to utilize low-cost nutrients for biomass culture; and 5) biomass productivity ≥ 0.10 g ash-free dry weight (afdw)/L.

3.3 Algal Culture

3.3.1 Indoor algal culture

Two culture media were used for isolation and subsequent growth comparisons: 1) Guillard's f/2 media (Guillard and Ryther, 1962) and 2) a combination of ammonium sulfate, pH adjusted phosphoric acid, and iron sulfate (ODI) (Richmond, 2003). In the case of diatomaceous species, the nutrient profile of the ODI medium was modified by addition of sodium silicate (Na_2SiO_3-$9H_2O$). Inoculum from 5.0-mL stock culture isolates was subsequently transferred into static 125-mL vials then to 1.0-L flasks outfitted with a stir bar. Those 1.0-L cultures that achieved productivity levels ≥ of 0.10 g/L ash-free dry weight (afdw) per day in f/2 medium were subsequently evaluated for growth on ODI medium that consisted of a standard nutrient blend of 2.0 mM nitrogen (N) from ammonium sulfate, 0.13 mM phosphorus (P) from pH balanced phosphoric acid, and 0.07 mM iron from iron sulfate at a 16:1 N:P ratio (Redfield, 1958). In order to evaluate ability to grow on ODI media, cultures successfully reared in f/2 were serially diluted once weekly at rates of 20, 25, 50, and 75% ODI medium as Guillard's f/2 is generally cost prohibitive for large scale production. All indoor samples were maintained at 25^{o}C, and exposed to a 24-hour photoperiod. Those candidates that did not achieve adequate productivity under these conditions were subsequently eliminated.

Cultures of selected species/strains were transferred to 20-L carboys that were inoculated with 100% ODI and were mixed with ambient aeration via 2cm airstone. Those that achieved ≥ 0.10 g/L were then transferred to ~400-L translucent cylinders (Solar Components Corporation, Manchester, NH). Each 400-L tank was outfitted with two 5-cm air stones and injected with ambient air for vertical mixing of culture water. Culture pH was determined using a Pinpoint TM pH probe (American Marine Inc. Ridgefield, CT) and adjusted to maintain desired pH using an

ASCO Red Hat ™ solenoid-controlled CO_2 delivery system (Grainger, Corpus Christi, TX).Water used to prepare cultures was adjusted to 28 ± 1ppt salinity using dechlorinated and filtered (diatomaceous earth) seawater from the Laguna Madre (near Corpus Christi, TX) and mixed with dechlorinated (15 ppm) municipal water. All large-volume cultures were monitored daily for temperature, salinity, and contamination (e.g., undesired species).

3.3.2 Outdoor algal culture

Outdoor raceways were filled with the algal inoculum from the indoor 400-L cylinders and treated seawater chlorinated to 15 ppm and diatomaceous earth filtered (Pentair Pool Products, Sanford, NC) prior to use. Upon achieving a productivity level of ≥ 0.15 g afdw/L, inoculum was transferred into 557-L raceways to achieve an initial biomass of ~ 0.15 g afdw/L at 5 cm depth. The water depth was increased in increments of 5 cm to a maximum depth of 20 cm. ODI media was added proportionally as depth increased. Mixed culture experiments were stocked with ~1/3 of each species. Each raceway production unit was fitted with a paddlewheel and longitudinal partition to maintain a water circulation velocity of ~50 cm/sec. Culture pH was monitored and adjusted as described above for indoor cultures. Nutrient medium (Ammonium sulfate, phosphoric acid, iron sulfate, and sodium silicate) was at various times to maintain productivity ≥ 0.10 g afdw/L. Upon achieving ~0.10 g afdw/L, outdoor raceways were partially harvested and biomass collected.

Temperature, salinity (YSI 2030, Yellow Springs International, Yellow Springs, OH, USA), and pH (EcoSense 100A, Yellow Springs International, Yellow Springs, OH, USA) were monitored daily. Productivity was monitored daily by obtaining samples from raceways in a 100mL Nalgene bottle daily. Biomass (TSS/VSS) analysis was performed according to Standard

Methods as outlined in APHA (1998). Daily TSS/VSS measurements (g/m^2) were used to calculate daily productivity over the course of the trials.

Upon achieving a productivity level ≥ 0.10 g afdw/L, raceways were partially harvested at ~418-L every two to four days in order to maintain productivity levels above 0.10 g/L afdw. Each treatment tank was drained into an adjacent 2.44 m diameter cylindrical tank for harvesting and dewatering using a BEAST centrifuge (WVO Designs, Charleston, SC, USA). Harvested biomass was retained for potential use in subsequent digestibility and growth trials. Additionally, four subsamples from each treatment (pre-dewatering) were collected and dewatered by centrifuge at 1,370 x g (Labconco, Kansas City, Missouri, USA) for 10 minutes and stored at -20^{o}C for further analysis.

3.4 Nutrient analysis

Moisture was determined after oven drying at 105^{o}C to constant weight and ash was quantified after combustion in a muffle furnace (Thermo Fisher Scientific, Richardson, TX, USA) at 550^{o}C overnight. The Dumas method (Ebeling, 1968) was used to determine crude protein (N × 6.25) following acid hydrolysis. Amino acids were evaluated using the HPLC method (AOAC, 1990). Crude lipid was determined gravimetrically (Folch et al., 1957) The gross energy content of algae was measured by combustion in a bomb calorimeter (Parr Instrument Company, Moline, IL, USA) using a benzoic acid standard (Schlosser et al., 2005).

3.5 Statistical analyses

Water quality variables (e.g., dissolved oxygen (mg/L), salinity (ppt), temperature (^{o}C), and pH and were recorded throughout the trials. Biometric factors (e.g., maximum biomass productivity, mean biomass productivity) within and between seasons for various species and

species mixes were tested for normality prior to running ANOVA ($P \leq 0.05$). When significant differences were identified among means for environmental or biometric data, they were compared using Tukey's test for multiple comparisons with a 95% confidence level. All statistical analysis was performed using the microcomputer software package R (5.3.1; Feather Spray, The R Foundation).

4. Results

4.1 Selection of algal species

Two dozen algal species were evaluated as potential candidates for biomass production in south Texas (Table 3).

Table 3. List of algal genera collected and evaluated as candidates for digestibility and growth trials as potential fishmeal replacements in the diet of *O. mossambicus.* The right-hand column below reflects the largest size culture achieved before failure or biomass harvest.

Algal Identification	Culture testing success
Amphidinium sp.	1-L flask[7]
Amphora sp.*	Raceway[4]
Chaetocerous sp.	40L cylinder[2]
Chlorella sp.	400-L cylinder[2]
Cryptomonas sp.	20-L carboy[6]
Cyanobacteria	Pre-dried material[9]
Cyclotella sp.	1-L carboy[6]
Cylindrotheca sp.	Raceway[2]
Dunalliella salina	1-L flask[8]
Gymnodinium sp.	1-L flask[8]
Isochrysis sp.	20-L carboy[5]
Microchloropsis salina	Raceway[8]
Navicula sp.	1-L flask[3]
Oscillatoria sp.	20-L carboy[3]
Phaeodactylum tricornutum	Raceway[8]
Platymonas sp.	Raceway[4]
Prochlorococcus marinus	125-mL flask[8]
Pseudonitzschia sp.	Failure to isolate[3]
Rhodomonas sp.	1-L flask[1]
Skeletonema oculata	Failure to isolate[1]
Synechococcus sp.	125-mL flask[8]
Tetraselmis sp.	400-L cylinder[5]
Thalassiosira sp.	20-L carboy[4]
Ulva sp.	125-mL flask[7]

[1] Nueces Bay
[2] Corpus Christi Bay
[3] Redfish Bay
[4] Packery Channel
[5] Laguna Madre
[6] Oso Bay
[7] Paul V. Zimba Lab – Texas A&M University – Corpus Christi (Center for Coastal Studies)
[8] UTEX culture collection – University of Texas Austin
[9] Global Algae Innovations – Lihue, HI.
*Intentionally isolated and cultured indoors from natural introduction in outdoor raceway

4.2 Algal cultures summer 2016

Results of outdoor culture trials for summer 2016 are shown in Tables 4-6. Water quality variables were appropriate for the growth and survival of *M. salina*, *Cylindrotheca* sp., and Summer mix 2016 (*Microchloropsis salina*, *Cylindrotheca* sp., *Phaeodactylum tricornutum*).

There were no significant differences ($P \geq 0.05$) in overall maximum and mean productivity for the duration of the trial after culture in summer 2016 (Table 5). However, *P. tricornutum* was significantly different ($P = 0.003$) from all other treatments due to its inability to survive past culture day 5. Of the remaining cultures, *Cylindrotheca* sp. had the lowest productivity (g/afdw/m^2/day) overall (40.18 ± 20.45) but was not significantly different from the *M. salina* (49.43 ± 23.59) and the Summer mix 2016 (42.29 ± 20.77).

Table 4. Mean environmental variables for outdoor algal cultures grown in summer 2016 (n=3), by treatment.

Outdoor Parameters	Summer mix 2016[1]	*M. salina*	*P. tricornutum*	*Cylindrotheca* sp.
Water temp (°C) A.M.	21.20 ± 1.74	20.78 ± 4.05	20.73 ± 4.04	20.73 ± 4.05
Water temp (°C) P.M.	24.97 ± 2.73	26.08 ± 4.14	26.01 ± 4.71	26.10 ± 4.14
Salinity (ppt)	28.80 ± 2.00	28.84 ± 2.28	28.66 ± 1.98	28.98 ± 2.19
pH	7.68 ± 0.17	7.45 ± 0.27	7.58 ± 0.25	7.53 ± 0.23

[1]*M. salina*, *Cylindrotheca* sp., and *P. tricornutum.*

Table 5. Maximum and mean daily productivity of outdoor algal cultures grown in summer 2016 (n=3), by treatment (g/afdw/m^2/day). Means with similar superscript in the same column are not significantly different ($p \leq 0.05$).

Productivity*	Summer mix 2016[1]	*M. salina*	*Cylindrotheca* sp.
Maximum Productivity (per treatment)	15.52	16.74	13.61
Mean Productivity	11.71 ± 3.36[a]	13.10 ± 2.98[a]	10.47 ± 2.62[a]

[1]*M. salina*, *Cylindrotheca* sp., and *Phaeodactylum tricornutum*

**P. tricornutum* not listed due to collapse of culture on the 5th day of the raceway trial.

Table 6. Overall productivity of outdoor algal cultures grown in summer 2016 (n=3), by treatment. Means with similar superscript in the same column are not significantly different ($p \leq 0.05$).

Algal Culture	Productivity (g/afdw/m^2/day)
Summer mix 2016[1]	42.29 ± 20.77[a]
M. salina	49.43 ± 23.59[a]
Cylindrotheca sp.	40.18 ± 20.45[a]
P. tricornutum	17.89 ± 11.86[b]

[1]*M. salina*, *Cylindrotheca* sp., and *P. tricornutum*.

4.3 Fall 2016 algal cultures

Results of fall 2016 are shown in Tables 7-9. Water quality variables were within appropriate limits for the growth and survival of Fall mix 2016, *M. salina, Playmonas* sp., and *Cylindrotheca* sp. (Table 7). There were no significant differences in overall maximum and mean biomass for the duration of the trial period (Table 8). *Cylindrotheca* sp. had a lower productivity from the other cultures but was not significantly different (P = 0.833). Mean biomass productivity was lower in the fall than the summer for all cultures.

Table 7. Mean environmental variables for outdoor algal cultures grown in fall 2016 (n=3), by treatment.

Outdoor Parameters	Fall mix 2016[1]	*M. salina*	*Platymonas* sp.	*Cylindrotheca* sp.
Water temp (°C) A.M	20.72 ± 4.04	20.78 ± 4.05	20.73 ± 4.04	20.73 ± 4.05
Water temp (°C) P.M.	28.28 ± 4.13	26.08 ± 4.14	26.01 ± 4.71	26.10 ± 4.14
Salinity (ppt)	28.84 ± 2.05	28.84 ± 2.28	28.66 ± 1.98	28.98 ± 2.19
pH	7.60 ± 0.25	7.45 ± 0.27	7.58 ± 0.25	7.53 ± 0.23

[1]*Microchloropsis salina*, *Platymonas* sp., and *Cylindrotheca* sp.,

Table 8. Maximum and mean daily productivity of outdoor algal cultures grown in fall 2016 (n=3), by treatment (g/afdw/m^2/day). Means with similar superscript in the same column are not significantly different ($p \leq 0.05$).

Productivity	Fall mix 2016[1]	*M. salina*	*Platymonas* sp.	*Cylindrotheca* sp.
Maximum Productivity (per treatment)	10.39	10.97	10.17	15.42
Mean Productivity	9.24 ± 4.96[a]	10.11 ± 6.48[a]	8.87 ± 3.97[a]	9.05 ± 8.73[a]

[1]*Microchloropsis salina*, *Platymonas* sp. and *Cylindrotheca* sp.

Table 9. Overall productivity of outdoor algal cultures grown in fall 2016 (n=3). Means with similar superscript in the same column are not significantly different ($p \leq 0.05$).

Algal Culture	Productivity (g/afdw/m^2/day)
Fall mix 2016[1]	10.77±7.40[a]
M. salina	10.90± 8.10[a]
Platymonas sp.	11.07±7.59[a]
Cylindrotheca sp.	8.69±6.15[a]

[1] *Microchloropsis salina, Platymonas* sp. and *Cylindrotheca* sp.

4.4 Biochemical composition of algal cultures

Dry matter content of outdoor cultures of algae ranged from a high of 95.94 ± 1.26% (sumix 2016) to a low of 86.73 ± 3.01% (fallmix 2016) (Table 10). The sumix 2016 outdoor cultures contained the lowest amount of mean protein (45.33 ± 0.11%). The greatest percent protein was provided by the cyanobacterial biomass sample (64.54 ± 0.66%). Cyanobacteria and *M. salina* (62.17 ± 0.84%) ingredient biomass contained the highest concentration of protein. The crude protein content of the cyanobacteria was higher than what is reported for white FM, ~ 64.5% and 62%, respectively (NRC, 2011).

Although the essential amino acids (EAA) of these sources were inferior to that of both soybean meal (SBM) and fishmeal (FM) they possessed amino acid profiles conducive to their inclusion in diets for subsequent tilapia digestibility and growth trials. In regards to Lys, the cyanobacteria content was comparable to that found in *Platymonas* sp., and sumix 2016. Crude protein content of all algal biomass investigated was higher than the average content of de-hulled SBM (~45%) (NRC, 2011). With few exceptions, FM and SBM essential amino acid (EAA) values were higher than all ingredient values. However, Lys was the same in the *Cylindrotheca* sp. ingredient (6.40%) to that of SBM and both were lower in Lys than FM (7.91%). Met content in cyanobacteria was numerically similar to that of SBM (1.35% and 1.30%, respectively). Percent Met in each algal culture widely ranged from (0.51% to 1.35%) in the *Cylindrotheca* sp. and the cyanobacteria, repectively. The cyanobacteria contained the least amount of Lys (2.23%) while

the fall mix 2016 (6.94%) was the closest to that of fishmeal reported in Ramierz et al., 2013 (7.91%). Additonally, the fall mix 2016 Lys content was higher than soybean meal (Alaski et al., 2013).

Table 10. Proximate analysis of screened outdoor algal cultures at Texas A&M AgriLife Research Flour Bluff (% dry weight) (n=2).

Algal Cultures	Dry Matter	Protein	Lipid
Fall mix 2016[1]	86.73 ± 3.01	60.70 ± 0.14	15.33 ± 0.02
M. salina	91.44 ± 2.22	62.17 ± 0.84	18.58 ± 0.01
Platymonas sp.	94.17 ± 1.77	49.82 ± 0.17	10.05 ± 0.01
Cylindrotheca sp.	94.60 ± 1.16	52.68 ± 0.37	13.52 ± 0.00
Spring mix 2014[2]	90.29 ± 2.56	55.12 ± 0.13	16.66 ± 0.01
Cyanobacteria[3]	92.96 ± 1.91	64.54 ± 0.66	10.36 ± 0.02
Summer mix 2016[4]	95.94 ± 1.26	45.33 ± 0.11	17.00 ± 0.06

[1]*Microchloropsis salina*, *Cylindrotheca* sp., *Platymonas* sp.
[2]*Microchloropsis salina*, *Cylindrotheca* sp., and *Amphora* sp.
[3]Cyanobacteria - Unknown culture location or method. Global Algae Innovations (Lihue, HI, USA)
[4]*Microchloropsis salina*, *Cylindrotheca* sp., and *P. tricornutum*.

Table 11. Amino acid composition of algal test cultures (% dry matter).

Amino Acids	Spmix 2014[3]	Sumix 2016[4]	Fallmix 2016[5]	*M. salina*	Cylindro[6]	Platy[7]	Cyano[8]	FM[*]	SBM[**]
Arg[1]	1.92	3.16	1.61	2.01	1.44	3.42	3.68	5.70	7.20
Cys[2*]	1.33	1.22	0.62	0.79	0.93	0.13	0.18	-	1.60
His[1]	0.86	0.67	0.68	0.84	0.67	0.81	1.18	2.41	2.60
Ile[1]	1.73	1.22	1.26	1.73	1.20	1.27	2.63	4.74	4.00
Leu[1]	4.39	3.35	3.37	4.34	3.32	4.27	4.88	7.74	7.80
Lys[1]	6.02	2.58	6.94	5.73	6.40	2.53	2.23	7.91	6.40
Met[1*]	0.88	0.68	0.66	0.79	0.51	0.75	1.35	3.02	1.30
Phe[1]	1.82	2.34	1.90	1.91	1.99	2.13	3.11	4.12	5.00
Tau[2]	0.00	0.20	0.30	0.00	0.28	0.00	0.00	-	-
Thr[1]	1.79	1.45	1.44	1.66	1.38	1.98	2.98	4.37	4.00
Val[1]	2.70	1.96	1.99	2.68	1.94	1.93	3.24	5.43	4.80

Values shown are from a single subsample (n = 1).
[1] Essential amino acid
[2] Non-essential amino acid
[3] Spring mix 2014 – *M. salina, P. tricornutum,* and *Amphora* sp.
[4] Summer mix 2016 – *M. salina, Cylindrotheca* sp., and *P. tricornutum*
[5] Fall mix 2016 – *M. salina, Cylindrotheca* sp., and *Platymonas* sp.
[6] *Cylindrotheca* sp.
[7] *Platymonas* sp.
[8] Cyanobacteria
* Ramierz et al., 2013
** Alaski et al., 2013

5. Discussion

In outdoor culture trials undertaken as part of this study, there were no significant differences in algal productivity within seasons. Previous studies have shown that fish and shrimp diets supplemented with *M. salina* resulted in higher growth rates and better protein retention efficiencies in the animals when compared to a soybean meal-based diet (Gbadamosi and Lupatsch, 2018). Other studies have shown that mixed cultures containing *M. salina* could replace up to 10% crude protein from fishmeal and soy protein concentrate without causing reductions in the growth performance of red drum (Patterson and Gatlin, 2013). *Microchloropsis salina* is

known to be productive in outdoor cultures (Ma et al., 2014), and have been proposed for the commercial production of eicosapentaenoic acid (EPA) (Apt and Behrens, 2009)

Certain species of cyanobacteria are well accepted as a feed ingredient in finfish (Patterson and Gatlin, 2013) and have been found to constitute a significant percentage of the natural diet of *Oreochromis* spp. (Getachew and Fernando, 1989; Zengeya et al., 2007) while others can be toxic (Zhou et al., 2006). Although the species composition of cyanobacteria used in this study is not known, many genera have the capability of producing considerable biomass (Ullah et al., 2015). The cyanobacterial biomass used in the present study contained a higher concentration of Met compared to the other algal cultures (see Table 11) as well as soybean meal, but had lower Met content than fishmeal. The concentration of other EAA were also much lower than that of FM and SBM, indicating a need for EAA supplementation in grow-out diets.

The dry ingredients evaluated in this study were chosen based upon results from previous studies. The indoor cultures were evaluated in temperate environments, with moderate salinity, stable dissolved oxygen, and controlled pH to determine their suitability for use as a potential fishmeal replacement. Studies have shown that the composition of macronutrients in algae can be selected for desired attributes by utilization of different nutrients (Lahaye et al., 1995; Menendez et al., 2002), or by altering metabolism, environmental stressors, or other abiotic factors (Rhyther et al., 1985; Keesing et al., 2016). Therefore, these genera should be evaluated for use in climates similar to those of south Texas. A marine species, with high protein, lipid, and daily biomass production has the potential to be beneficial to both the biofuel and feed industry. Therefore, further evaluation is warranted.

Overall, all cultures performed well in outdoor algal growth trials and although the EAA of these sources were inferior to that of both SBM and FM they possessed amino acid profiles

conducive to their inclusion in diets for subsequent tilapia growth trials. The only exception was *P. tricornutum* which did not survive the summer 2016 trial and was terminated early in the outdoor culture phase. However, other research indicates that *P. tricornutum* grows well in the cooler seasons in south Texas. Had the growth trial been performed in the early spring, adequate biomass might have been obtained.

CHAPTER III: EVALUATION OF MARINE MICROALGAE DIGESTIBILITY IN OREOCHROMIS MOSSAMBICUS (MOZAMBIQUE TILAPIA) DIETS

Abstract

Unicellular marine microalgae were isolated from natural waters surrounding Corpus Christi, TX and cultured outdoors during spring 2014, summer 2016, and fall 2016. Additionally, *Microchloropsis salina* and cyanobacteria sp. were acquired from outside sources for use as part of a comprehensive study to identify algal species/mixes as potential source of proteins for *Oreochromis mossambicus* diets.

Forty ~30-g juvenile tilapia were placed into 830-L tanks (n=4) to evaluate the digestibility of proximate components and essential amino acids of test ingredients. Experimental diets were composed of 69% reference diet composed of typical feedstuffs, 1% Cr_2O_3, and 30% algal ingredient (*Platymonas* sp., *Cylindrotheca* sp., cyanobacteria, *M. salina,* a mixture of *M. salina, P. tricornutum,* and *Ampora* sp, and a mixture of *M. salina, Cylindrotheca* sp., and *Platymonas* sp.).

Selection of algae for digestibility trials involved evaluation of test ingredients for apparent dry matter (DM), protein, and amino acid digestibility when fed to juvenile (~30g) tilapia. Apparent dry matter digestibility ranged from 50.7±0.02% to 70.6±0.07% in the *M. salina* and Spring mix 2014 (Spmix), respectively. The *Platymonas* sp. and the Spmix diets exhibited the highest dry matter digestibility (69.3±5.5 and 70.6±3.9%, respectively). The dry matter in the *Cylindrotheca* sp. (57.9±3.5%) and the *M. salina* (50.7±8.5%) diets were the least digestible. Results showed that there were no significant differences in regards protein digestibility in the formulated diets (n=3). Also, there were no significant differences between the digestibilities of the ingredients (ADCI). The *Platymonas* sp. and the Spring mix 2014 had the highest ADCI

83.1±30.0% and 85.3±24.6%, respectively. Methionine was 93.0% digestible in *M. salina*, and lysine digestibility was highest in Spmix (88.5±0.46%). Results suggest that *Platymonas* sp., and Spmix should be examined further as a potential fishmeal replacement in juvenile tilapia diets.

1. Introduction

The protein content and amino acid composition of protein feedstuffs and the degree to which they are utilized to meet the metabolic needs of a given animal determines the nutritional quality of those feedstuffs. However, the quality of an aquaculture diet is not based purely on its overall biochemical composition. Aquaculture feeds are typically composed of several dietary ingredients and often contain a variety of proteins of dissimilar nutritive value, ultimately affecting biological availability of amino acids (AA) (Figueiredo-Silva et al., 2015).

Numerous factors contribute to the ability of the animals to digest and absorb nutrients. Stone (2003) showed that fish from a lower trophic level, such as tilapia, are more efficient at degrading carbohydrates than higher trophic level fish, such as salmon. This is likely due to the longer gut, in relation to carnivorous fish, resulting in increased time for digestion or higher surface area for absorption (Uscanga et al., 2010). This is supported by research that showed that *Arthrospira* sp. and *Chlorella* sp. was 94-95% digestible by Nile tilapia but only 79% by Caspian great sturgeon (Safari et al., 2016; Sarker et al., 2016). Therefore, differences in protein digestibilies may be due to differences in the properties of the proteins or cell wall matrices (Teuling et al., 2017). Also, fish species (Teuling et al., 2017), feeding habits, developmental stage of the fish, environmental temperature (Refstie et al., 2006), nutritional requirements of the animal (Santos et al., 2013), and health of the fish are all factors that contribute to nutrient utilization. Additionally, it is also important to determine the processing of ingredients, location of plant culture or farm, time of year

the ingredient was harvested, as well as methods used for evaluation and feces collection to make a direct comparison of the results of each study. Due to the above referenced factors, results from digestibility studies typically show high variability.

Dozens of feed studies have been conducted with tilapia fed different species of algae as a protein replacement and using either performance indices and/or apparent digestibility coefficients when including microalgae as a soybean meal, fishmeal, or fish oil replacement. However, microalgae has been primarily evaluated as a microfeed ingredient with the focus of overall beneficial properties instead of gross nutrients available to the animal (Shah et al., 2018). For example, El-Sayed et al. (2013), and Rincon et al. (2012) used varying levels of *Arthrospira* spp. in the diet of tilapia. These studies reported no change to better survival and growth when microalgae was used as a replacement in fishmeal at levels of 30% to 43%, respectively. Another study showed that 100% of fish oil could be replaced with *Schizochytrium* sp. in the diet or *Oreochromis niloticus*, resulting in improved weight gain, FCR, and PER (Sarker et al., 2016). Therefore, the primary objective of this study was to evaluate the digestibility of various algal ingredients screened from diverse culture environments (Chapter II) to further evaluate algal test ingredients for replacement of fishmeal in tilapia diets.

2. Methods

2.1 Biochemical composition of algal test ingredients and reference diet.

Biochemical composition of the algal test ingredients are shown in chapter II. Moisture was determined after oven drying at 105^{o}C to constant weight. Ash was quantified after combustion in a muffle furnace (Thermo Fisher Scientific, Richardson, TX, USA) at 550^{o}C overnight. The Dumas method (Ebeling, 1968) was used to determine crude protein (N × 6.25)

following acid hydrolysis. Amino acids were evaluated using the HPLC method (AOAC, 1990). Crude lipid was determined gravimetrically (Folch et al., 1957) The gross energy content of algae was measured by combustion in a bomb calorimeter (Parr Instrument Company, Moline, IL, USA) using a benzoic acid standard (Schlosser et al., 2005). The Kjeldahl method (Kirk et al., 1950) was used to determine apparent digestibility coefficients of diets, ingredients, and nutrients. Resulting data was used to develop formulation for the experimental diets.

2.2 Ingredient composition of reference and test diets

The reference diet was formulated using Concept 5 Feed Formulation Software (CFC Tech Services Inc., Pierz, MN, USA). Experimental diets contained 69% (wet weight) reference ingredients, 30% algae ingredient, and 1% chromic oxide.

Table 12. Ingredient composition of reference diet. (dry wt. basis). Sources of material are listed in the footnotes.

Ingredients	g kg^{-1}
Casein[1]	43.0
Menhaden Fish Meal (62%)[2]	100.0
Soybean Meal[3]	450.0
Dextrin[4]	250.0
Cr_2O_3[5]	10.0
DL-MET[6]	3.0
Alginate[7]	20.0
Cellulose[8]	10.0
Menhaden Fish Oil[9]	20.0
$CaH_4P_2O_8$[10]	10.0
Vitamin Premix[11]	30.0
Mineral Premix[12]	40.0
Corn Oil[13]	14.0

[1] Sigma Aldrich, St. Louis, MO, USA
[2] Zeigler Bros. Inc., Gardners, PA, USA
[3] Producers Cooperative Association, Bryan, TX, USA
[4] MP Biomedicals, Solon, OH, USA
[5] Acros Organics, NJ, USA
[6] Alfa Aesar, Reston, VA, USA
[7] Acros Organics, NJ, USA
[8] Alfa Aesar, Reston, VA, USA
[9] Zeigler Bros. Inc., Gardners, PA, USA
[10] PCS Sales, Joplin, MO, USA
[11] Zeigler Bros. Inc., Gardners, PA, USA
[12] Zeigler Bros. Inc., Gardners, PA, USA
[13] ACH food companies, Cordola, TN, USA

2.3 Feed preparation

Screened algal test taxon/mixtures are listed in Chapter II. Test diets were prepared in 1.3-kg batches in the following manner: Dry test ingredients were ground using a burr mill (Mr. Coffee Automatic Burr Mill, Neosho, MI, USA) and passed through a mesh sieve with ~500 µm pore size before use in diet preparation. Dry ingredients were homogenized in a food mixer (Model A-200, Hobart Corporation, Troy, OH, USA) for 30 minutes. Menhaden fish oil was then added and mixed for an additional 15 minutes. An alginate binder was added to deionized water (400 mL kg^{-1} diet)

in a separate bowl and blended using a hand mixer for approximately 45 seconds. The resulting mixture and warm deionized water (300 mL kg^{-1}) were added to the ingredients and mixed for another 15 minutes to obtain the appropriate consistency for mash extrusion. A meat chopper (Model A-800 Hobart #12, Hobart Corporation, Troy, OH) fitted with a 3-mm die was used for extrusion of moist feed strands. These were separated and dried on wire racks in a forced air convection oven at 40°C until reaching ~8-10% moisture content. The feed was then ground to the appropriate size for fish consumption, sifted, bagged, and stored at 4°C.

2.4 Research facility and acclimation

Oreochromis mossambicus (tilapia) were obtained as juveniles from Larry's Fish Farm, Giddings, Texas, USA. The fish were stocked into 5-m outdoor holding tanks at the Texas A&M AgriLife Research Mariculture Facility in Flour Bluff, TX, USA. Fish were held in fresh dechlorinated domestic water that had been passed through a pressurized sand filter and introduced to tanks at a flow rate of 1.9L/min/tank (~500% exchange per day). Juveniles were hand-fed a commercial tilapia production diet (Ziegler 35%, protein, 6% lipid; Gardeners, PA, USA), three times daily until satiated and grown until achieving a mean individual weight of approximately 30g.

2.5 Stocking of Treatment Tanks

Fish were randomly selected from outdoor holding tanks, described above and stocked as groups of 30 individuals per tank (mean initial wt. of 26.5 ± 3.5 g). Culture tanks were part of a recirculating aquaculture system of 0.71 m in depth with a bottom area of 1.17 m^2 and contained 830 L of water. Juvenile tilapia were acclimated to system conditions over a 7-day period during

which they were fed the above referenced diet at 3% bwd (feed per wet biomass weight of fish per day). Fish were subjected to a 24-h fasting period prior to initiation of experiments.

2.6 Digestibility Trial Management

Treatment tanks (n = 4) were assigned according to a randomized block design, and feed was manually offered at a rate of 3% bwd (Adeoyea et al., 2016) two times daily at 9:00 am and 4:00 pm (Wang et al., 2017) for 3-days with a commercial diet (Zeigler Bros. 35% protein, 6% lipid tilapia diet) before trial initiation. Prior to addition of digestibility feeds, all tanks were siphoned to remove uneaten feed and feces. Fecal samples were collected by siphon 13-h post addition of experimental feeds and collected into individual 50-mL Falcon tubes. Feces were allowed to settle in tubes and water removed by pipette. Fecal samples were pooled over a period of 3 days by treatment and stored at -20 °C until being lyophilized for nutrient analysis.

Water temperature (°C), salinity (ppt), dissolved oxygen (mg/L) were measured daily in one tank from each treatment with a YSI 85 oxygen/conductivity instrument (YSI, Yellow Springs, Ohio, USA). Daily analyses of NH_3, NO_2, and NO_3 were performed using test strips (Tetra 6-1 EasyStrips and Tetra Ammonia Easy Strips, Blacksburg, VA, USA). and were also evaluated once weekly using a Hach DR/2100 spectrophotometer (Hach, Loveland, CO, USA).

2.7 Determination of digestibility coefficients

The following equations were used to calculate digestibility coefficients for dry matter (DM), ingredient, and nutrient digestibility:

1) $\%\ ADC_{DM}{}^{1} = (1 - (Cr_2O_3\ diet/Cr_2O_{3\ feces})) \times 100$

2) % ADC $_{nutrient}$[2] = 100% (($Cr_2O_{3\ feed}$/$Cr_2O_{3\ feces}$) (Nutrient content $_{feces}$/Nutrient content $_{feed}$))

3) % ADC $_{ingredient}$[3] = ((ADC $_{test\ diet\ -}$ (0.7 × ADC $_{reference\ diet}$)) /0.3)

[1] (Forster, 1999)
[2] (Bureau, 1999)
[3] (Cho et al., 1982)

2.8 Statistical analyses

Digestibility results are presented as mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA, $\alpha = 0.05$) using R (5.3.1; Feather Spray, The R Foundation) statistical package for Windows. When significant differences were identified, means were compared using a Tukey test for multiple comparisons with a 95% confidence level. Results from water quality determinations were reported as minimum, maximum, and mean ± standard deviation and standard error.

3. Results

During the digestibility trial, all water quality factors were within the range for normal growth and survival of *Oreochromis* spp. (El-Sayed, 2006a; 2006b) and water quality among the various treatments (Table 13) was appropriate for the growth and survival of *O. mossambicus*.

There were no statistical differences in dry matter in the formulated diets (92.66 – 94.71%). *Cylindrotheca* sp. contained the least amount of ash (1.76 ± 0.22%) and was significantly different from all other diets ($P = 0.006$). There were no significant differences in the levels of protein

between diets. The crude lipids were significantly different with *Platymonas* sp. containing the largest percentage (14.68 ± 0.06), and the reference diet having the least (6.46 ± 0.37).

The Spring mix 2014 had the highest levels of arginine (Arg) (3.63), lysine (Lys) (5.44), methionine (Met) (1.90), valine (Val) (4.48), isoleucine (Ile) (3.66), and leucine (Leu) (4.94). The reference diet had the lowest level of Lys (2.95), and the *Platymonas* sp. diets amino acid digestibility was lowest in all AA other than Met (Table 14).

Table 13. Water quality means for the duration of digestibility trials (n=4).

Water quality variables	Mean
Water temperature (^{o}C)	26.33 ± 2.22
Salinity (ppt)	2.53 ± 0.14
pH	8.34 ± 0.18
D.O. (mg/L)	9.74 ± 0.71
NH_3 (mg/L)	0.29
NO_3 (mg/L)	0.80
NO_2 (mg/L)	0.09

Table 14. Proximate values of formulated feeds (%) (n=2).

Digestibility	Dry Matter	Ash	Protein	Lipid
Reference	92.66 ± 0.10	2.36 ± 0.34	33.68 ± 0.25	6.46 ± 0.37
Platymonas sp.	93.76 ± 0.49	2.21 ± 0.33	36.68 ± 0.12	14.68 ± 0.06
Cylindrotheca sp.	94.71 ± 0.05	1.76 ± 0.22	35.34 ± 0.97	9.02 ± 0.01
Spring mix 2014[1]	93.14 ± 0.07	2.30 ± 0.11	37.63 ± 0.33	13.52 ± 0.00
Fall mix 2016[2]	92.99 ± 0.22	2.86 ± 0.38	36.31 ± 0.05	10.88 ± 0.02
M. salina	93.91 ± 0.07	2.95 ± 0.18	37.15 ± 0.19	9.37 ± 0.00

[1] *M. salina, P. tricornutum, Amphora* sp.
[2] *M. salina, Platymonas* sp., *Cylindrotheca* sp.

Table 15. Amino acid composition of digestibility diets (n=2) (% crude protein). Means with similar superscript in the same column are not significantly different ($p \leq 0.05$).

Amino Acids	Reference	*Platymonas* sp.	*Cylindrotheca* sp.	Spring mix 2014[3]	Fall mix 2016[4]	*M. salina*
Arg[1]	3.37±0.04	2.34±0.05	3.36±0.23	3.63±0.35	3.36±0.28	3.35±0.19
Cys[2*]	4.10±0.00	3.90±0.00	4.70±0.00	3.50±0.00	4.20±0.00	4.20±0.00
His[1]	1.57±0.21	1.19±0.10	1.87±0.00	1.75±0.40	1.56±0.10	1.74±0.18
Ile[1]	2.67±0.05	2.04±0.10	2.55±0.15	3.66±0.27	2.86±0.29	2.76±0.19
Leu[1]	3.76±0.18	3.12±0.10	4.44±0.40	4.94±0.32	4.12±0.22	4.19±0.20
Lys[1]	2.95±0.07	3.63±0.11	4.31±0.18	5.44±0.40	4.72±0.28	4.78±0.22
Met[1*]	8.60±0.00	8.20±0.00	8.80±0.00	8.10±0.00	8.10±0.00	9.30±0.00
Phe[1]	3.41±0.13	2.63±0.07	2.78±0.11	4.25±0.23	2.64±0.17	3.63±0.19
Thr[1]	2.14±0.39	2.00±0.10	2.54±0.19	2.62±0.18	2.67±0.23	2.71±0.18
Val[1]	3.30±0.56	2.81±0.10	3.93±0.16	4.48±0.36	3.68±0.25	3.72±0.18

[1] Essential amino acid
[2] Non-essential amino acid
[3]*Microchloropsis salina, Phaeodactylum tricornutum, Amphora* sp.
[4]*Microchloropsis salina, Platymonas* sp., *Cylindrotheca* sp.
*Analyzed by New Jersey Feed Labs, Ewing Township, NJ, USA (n=1).

Apparent nutrient digestibility of diets

Apparent dry matter digestibilities of the reference and test diets are shown in Table 16. The *Platymonas* sp. and the Spring mix 2014 diets exhibited the highest dry matter digestibility (69.3 ± 5.5% and 70.6 ± 3.9%, respectively). The *Cylindrotheca* sp. (57.9 ± 3.5%) and the *M. salina* (50.7 ± 8.5%) were the least digestible of the diets in regards to dry matter. The crude protein in test ingredients ranged from 45.33 ± 0.11% (Summer mix 2016) to 64.54 ± 0.66% (cyanobacteria) (Chapter II). The apparent protein digestibility ranged from 80.2 ± 3.3% (*Microchloropsis salina*) to 88.2 ± 1.8% (reference). There were no significant differences within or between any of the diets in regards apparent protein digestibility. There were no signficant differences in AAAD in any of the other diets. There were no significant differences in apparent digestibility of any of the ingredients (ADCI). The ADCI of ingredients ranged from 18.1 ± 41.0% to 85.3 ± 24.6% (*M. salina* and *Platymonas* sp., respectively) (Table 17).

Table 16. Apparent digestibility of dry matter (ADDM), protein (ADP), and ingredient (ADCI) in *O. mossambicus* feed ingredients (n=3).

Diet	ADDM (%)	ADP (%)	ADCI (%)
Reference	63.4 ± 5.6	88.2 ± 1.8	63.4 ± 5.6
Platymonas sp.	69.3 ± 5.5[a]	85.6 ± 2.6[a]	83.1 ± 30.0[a]
Cylindrotheca sp.	57.9 ± 3.5[c]	85.7 ± 1.2[a]	44.4 ± 24.7[a]
Spring mix 2014[1]	70.6 ± 3.9[a]	88.0 ± 1.6[a]	85.3 ± 24.6[a]
Fall mix 2016[2]	63.4 ± 3.5[b]	87.5 ± 1.2[a]	63.1 ± 1.6[a]
M. salina	50.7 ± 8.5[c]	80.2 ± 3.3[a]	18.1 ± 41.0[a]

[1] *M. salina, P. tricornutum, Amphora* sp.
[2] *M. salina, Platymonas sp., Cylindrotheca* sp.

Table 17. Apparent digestibility (%) of amino acids in reference diet and test ingredients fed to juvenile *O. mossambicus* (n=2). Means with similar superscript in the same column are not significantly different ($p \leq 0.05$).

Amino acid	Reference	*Platymonas* sp.	*Cylindrotheca* sp.	Spring mix 2014[3]	Fall mix 2016[4]	*M. salina*
Arg[1]	84.8 ± 1.9	71.4 ± 1.6[a]	77.3 ± 5.5[a]	79.7 ± 7.3[a]	80.0 ± 5.1[a]	74.3 ± 5.6[a]
Cys[*2]	56.0 ± 0.0	38.0 ± 0.0	47.0 ± 0.0	46.0 ± 0.0	55.0 ± 0.0	40.0 ± 0.0
His[1]	83.8 ± 0.4	75.1 ± 2.7[a]	79.7 ± 0.0[a]	82.1 ± 7.6[a]	81.2 ± 1.8[a]	77.1 ± 4.7[a]
Ile[1]	87.0 ± 1.9	75.8 ± 2.8[a]	78.8 ± 4.3[a]	78.8 ± 3.7[a]	83.5 ± 4.4[a]	76.7 ± 5.2[a]
Leu[1]	75.4 ± 2.8	58.2 ± 4.8[a]	73.2 ± 3.6[a]	73.0 ± 6.1[a]	70.2 ± 6.1[a]	58.6 ± 9.4[a]
Lys[1]	82.7 ± 2.4	82.7 ± 2.3[a]	84.4 ± 3.2[a]	88.5 ± 4.6[a]	86.9 ± 3.3[a]	81.9 ± 4.6[a]
Met[*1]	86.0 ± 0.0	82.0 ± 0.0	88.0 ± 0.0	81.0 ± 0.0	81.0 ± 0.0	93.0 ± 0.0
Phe[1]	85.0 ± 1.8	71.4 ± 2.1[ab]	69.7 ± 4.0[b]	82.3 ± 4.0[ab]	73.7 ± 4.0[ab]	74.3 ± 5.4[ab]
Thr[1]	75.6 ± 0.3	69.2 ± 3.4[a]	71.7 ± 6.8[a]	74.3 ± 5.0[a]	75.8 ± 5.1[a]	67.4 ± 7.0[a]
Val[1]	83.8 ± 2.1	74.2 ± 3.0[a]	80.6 ± 3.5[a]	80.6 ± 6.1[a]	80.9 ± 4.5[a]	74.1 ± 5.5[a]

[1] Essential amino acid
[2]Non-essential amino acid
[3] *M. salina, P. tricornutum, Amphora* sp.
[4] *M. salina, Platymonas sp., Cylindrotheca* sp.
[*]Analyzed by New Jersey Feed Labs, Ewing Township, NJ, USA (n=1)

4. Discussion

Although a variety of factors affect the moisture content of the ingredient, the timing and method of harvest may have been a determining factor as crude protein concentration of algae is dependent upon their growth phase (Fernandez-Reiriz et al., 1989; Oregon State University, 2004; Whyte, 1987). Due to the harvest method requirements and laboratory limitations, harvest of the

algae cultures remained in holding tanks for varying amounts of time while dewatering was performed. Although each ingredient was thoroughly dried, the "holding time" may have resulted in a change of the composition of the cell itself. These changes may alter the amount of dry matter, crude protein, and AA availability in each ingredient. Unfortunately, a power outage following hurricane Harvey resulted in the decomposition of pre-harvest samples. Therefore, a comparison of nutrients could not be made between the initial harvest and post de-watering period.

Digestibility of dry matter varied between diets, but there were no significant differences between the APDs of the diets. In some studies, low protein digestibility has been associated to cell wall structure and accessibility of nutrients (Gong et al., 2017; Sarker et al., 2016; Teuling, 2017). In this study, intact algae was used in formulated fish diets.

There are a large diversity of cell wall structures that may limit digestion (Palinska and Krumbein, 2000; Scholz et al., 2014) in certain animals. Due to this, the protein and other nutrients found in a cell may not have direct contact with digestive enzymes. Numerous studies have been performed that focus on specifically on these enzymes and their ability to hydrolyze substrates between fish species (Applebaum and Holt, 2003; Cara et al., 2007; Papoutsoglou and Lyndon, 2006). Tilapia in particular have shown the capacity to digest microalgae by leaching nutrients from the cell wall without breaking its contents (Horn and Messner, 1992). Tilapia also have a reduced stomach size, and a gut pH of ~1 (Ekpo and Bender, 1989), allowing a large portion of the food to be primarily digested in the anterior part of the intestine (Uscanga et al., 2010). The combination of these mechanisms allow this omnivore effective methods to enhance the digestion of nutrients contained in algal cells. Digestibility of ingredients also varies between species of tilapia. In a study comparing absorptive intestinal surface area in two tilapia species, *Oreochromis*

aureus was found to have 21% greater digestive surface area greater than *tilapia zilli* (Frierson and Foltz, 1992).

Overall, apparent digestibility of the crude protein in all of the experimental diets were not significantly different from the reference diet (88.2 ± 1.8%). Although there were no significant differences between the digestibilities of the ingredients (ADCI), the *Platymonas* sp. and the Spring mix 2014 had the highest ADCI (83.1 ± 30.0% and 85.3 ± 24.6%, respectively). The ADCI of ingredients ranged from 18.1 ± 41.0% to 85.3 ± 24.6% (*M. salina* and *Platymonas* sp., respectively) (Table 17). The large standard deviation within the replicates likely affected these results. In the future, pooled fecal samples should be mixed more thoroughly to homogenize the sample before analysis. The *M. salina* ingredient contained a high crude protein content that exceeds reported values determined for soybean meal in terms of crude protein (~45% for high-pro soybean meal and 62.17% *Microchloropsis* meal) (Schneider et al., 2004). Compared to other test ingredients, the dried *Microchloropsis salina* biomass, if included in a production diet, would likely be used at an inclusion level much lower than that of other test ingredients. This ingredient should be further analyzed as it has been shown (Teuling et al., 2019) that strain differences, batch differences, or seasonal changes in the culture of *Nannochloropsis gaditana* result in varying levels of nutrient accessibility in *O. niloticus*. Although the Teuling (2019) used different methods, in that it was not performed by adding algal biomass to formulated diets, it was believed that *M. salina* would exhibit similar characteristics as *N. gaditana* in regards to digestibility. In this study, *M. salina* contained 80.2 ± 3.3% digestible crude protein. Therefore, it is recommended that studies examining nutrient composition be standardized for comparison between future digestibility trials.

Methionine digestibility of fishmeal in the diets of *Oreochromis* spp. has been reported to be as low as 87% (Hanley, 1987). However, in general, studies have shown that FM is ~93%

(Guimaraes et al., 2008; NRC, 2011; Watanabe et al., 1996) digestible in *Oreochromis* sp. One report shows a Met digestibility of only 84% in a diet formulated with solvent extracted SBM (NRC, 2011) while another showed Met in soybean meal (49% crude protein) to have a digestibility of (~93%) (Guimaraes et al., 2008). The *M. salina* diets Met values were approximately the same as the FM and SBM as reported in Guimares et al., (2008) and in NRC (2011). The reference (86%) and *Cylindrotheca* sp. (88%) Met digestibilities were higher than the 84% reported in the solvent extracted SBM study (NRC, 2011). The Lys content of SBM and FM in NRC (2011) are 83 and 91%, respectively. Lysine was highest in the Spring mix 2014 (88.5 ± 4.6%), and the two lowest were the reference diet (88.7 ± 2.4%) and the *Platymonas* sp. diet (82.7 ± 2.3%).

In this trial, the fish did not consume the cyanobacterial diet. Therefore, further examination of the digestibility of this ingredient was not performed. Hence, even though the nutritional value is similar to that of fishmeal, the digestibility is unknown. This potential feed ingredient should be re-examined perhaps with a different formulation containing chemoattractants or using alternative processing methods.

Little research has been performed on *Cylindrotheca* spp. as a component of aquaculture diets. Although growth and survival have been examined in abalone (Matsumoto et al., 2015, 2018), and sea cucumbers (Junwei et al., 2015) fed *Cylindrotheca closterium*, no other work on the diet of aquatic animals fed this ingredient could be identified for further evaluation of *Cylindrotheca* spp. as a dried feed additive.

In this study, digestibility values of each essential amino acids were within acceptable levels and the AA requirements for *Oreochromis* spp. (Jauncey, 1982; NRC, 2011; Michelato et al., 2016; Michelato et al., 2017) were met for all of the digestibility diets. Numerous studies have

focused on mainly health and growth parameters (Gong et al.,2017; Hussein et al.,2013; Vizcaíno et al., 2014; Walker and Berlinsky, 2011), but have produced dissimilar results. The available data on nutrient digestibility of unicellular algae in the diets of fish species are extremely limited. Therefore, digestibility of each ingredient must be examined in order to evaluate apparent digestibility coefficients (ADCs) of crude protein and amino acids. A larger sample size for future studies is suggested as this comparison does not suggest any difference in ingredient digestibility. Additionally, protein solubility, cell wall integrity, species of tilapia, and gut transit time of the various species of alga in the fish diet should be investigated in future research.

CHAPTER IV: EVALUATION OF MARINE MICROALGAE AS A FISHMEAL REPLACEMENT IN DIETS FOR *OREOCHROMIS MOSSAMBICUS* (MOZAMBIQUE TILAPIA).

Abstract

A 30-day feeding trial was performed utilizing *Platymonas* sp. (P) and spring mix 2014 (SM) (*Microchloropsis salina, Phaeodactylum tricornutum,* and *Amphora* sp.) as part of a comprehensive study to identify algal species/mixes as potential source of proteins and amino acids for inclusion in diets of *Oreochromis mossambicus*. Ten ~0.170 mg tilapia were fed diets in which SM and P replaced various levels of fishmeal (0, 20, 40, 60, 80, 100%) in diets containing 40% crude protein. Weight gain (%), specific growth rate (SGR), final body wt., feed conversion ratio (FCR), protein efficiency ratio (PER), and percent survival were evaluated (n=5). Survival ranged from 96-100%. There were no significant differences in percent weight gain in the P20%-P80% diets. The FCR of P20% and P40% were different from the P100% diet (P=0.0145). The PER of P20%, P40%, and P80% were also significantly different from P100% (P=0.0214) diet. The P100% diet had the lowest FCR of all of the diets (1.19±0.14). All performance indices were similar for tilapia fed the SM diets. Results showed that both test ingredients could be used to replace fishmeal at high levels of dietary inclusion, 80% for *Platymonas* sp. and 100% for SM. This indicates high potential for replacement of fishmeal in tilapia feeds with marine microalgae.

1. Introduction

Compounded feeds represent the principal cost component in intensive aquaculture production, representing over 50% of all operating expenses (El-Sayed, 2006a). For most commercial aquaculture species, fishmeal serves as the major traditional feed ingredient providing protein and essential amino acids (Ng and Romano, 2013). At present, fishmeal is expensive (~$1,500/MT; Index Mundi, 2019), has high market price volatility, and is known to negatively impact marine food webs (Olsen and Hasan, 2012; Tacon and Metian, 2009). The cost of fishmeal is expected to increase 90% by 2024, and these increased costs have resulted in the evaluation of many potential alternative feed ingredients such as soybean, barley, rice, peas, canola, lupine, wheat gluten, corn gluten, yeast, insects, and algae (Patterson and Gatlin, 2013; Salze et al., 2010; Schneider et al., 2004). From a feed manufacture perspective, reduction of dietary inclusion of marine animal meals could significantly reduce feed ingredient cost; however, less costly alternative ingredients must also provide similar overall benefit. From a purely nutritional perspective, the usefulness of replacement ingredients is largely dependent upon how well they can provide essential nutrients in meeting the nutritional requirements of the species in question. Additionally, concern about the competition for these ingredients from the human food industry (i.e., food security) either directly or indirectly by inclusion in other types of feeds (e.g., livestock and poultry) which provide nutrition to humans (El-Sayed, 2006a). Identifying "non-competitive" sources of protein and lipids is of paramount importance as many of these alternative ingredients are, by themselves, nutritionally deficient (Salze et al., 2010; Zhou et al., 2005).

The use of expensive ingredients (e.g. fishmeal) and its use in the feed manufacturing process make aquaculture feeds some of the most expensive (Furuya and Furuya, 2010). Replacement of fishmeal with alternative protein sources that contain suitable levels of essential

nutrients, are readily available, and low-cost, is crucial to reduce the use of fishmeal in diets. If identified, these ingredients could decrease overall cost of feed and fish production.

To evaluate marine algae as a source of protein and lipid in aquaculture feeds, it is necessary first to compare it to a more traditional, albeit unsustainable, nutrient source such as fishmeal. As a major nutrient in aquaculture feed, fishmeal contains a high (~65% dry matter) level of protein, is replete in marine essential marine fatty acids, and supplies substantial levels of vitamin E, minerals, and phospholipids. It also possesses potent chemoattractive qualities suitable for improving feeding efficiency in aquaculture feeds (NRC, 2011). Therefore, to serve as a replacement for fishmeal, alternative ingredients must be both nutritionally and economically comparable either as individual ingredients or as low-cost mixtures of ingredients.

Microalgae have been studied as an alternate nutrient source largely due to their highly variable physical and chemical attributes (Bennamoun et al., 2015). Variability in biochemical composition is affected by numerous factors, including species of algae, culture salinity, temperature, ambient light intensity, and nutrient availability (Sharma et al., 2012). These factors are often reflective of biotic and abiotic environmental conditions, geographic area, season, and life stage (Mabeau and Fleurence, 1993). Additionally, the methodologies of algae biomass production (e.g, culture vessel, method of introduction of nutrients, dewatering, and drying) can introduce variance. Algal biomass also has commercial applications such as food colorants, dyes, cosmetics, pharmaceuticals, pigments, toxins, pollution control, and food additives (Mata et al., 2010). Thus, algal biomass can provide value-added co-products (Suganya et al., 2016) that if produced for certain disciplines (e.g., algae biofuels industry) can improve cost margins. Because of this interest by various industries, aquatic plant production has increased 44% since 2012, with 30.1 million tonnes provided by aquaculture in 2016 (FAO, 2018).

Commercialization of marine microalgae may have further advantages in that its use of water for culture as it does not directly compete with water demand for domestic human consumption. Published research on the use of algal species as an aquafeed is becoming increasingly common. The primary focus to date is on freshwater species of algae that are novel, non-commercial, and produced in limited supply (Ng and Romano, 2013). Due to their tremendous diversity and capability for high productivity, marine species of microalgae capable of being commercially mass-produced also warrant evaluation for use in aquaculture feeds (FAO, 2016).

The primary objective of this study was to evaluate the effect of fishmeal replacement with algae on weight gain (%), survival (%), specific growth rate (SGR), feed converstion rate (FCR), and protein efficiency ratio (PER) of *Oreochromis mossambicus*.

2. Methods

2.1 Algal ingredients

Platymonas sp. and Spring Mix 2014 (SM) were both cultured outdoors at Texas A&M AgriLife – Flour Bluff in the spring of 2014 as well as the fall of 2016, respectively. The SM consisted of three species of algae *Microchloropsis salina*, *Phaeodactylum tricornutum*, and *Amphora* sp. Collection methods, isolation methods, and nutrient values of all algal ingredients are reported in Chapter II. Both algal cultures met the selection criteria regarding crude protein content and growth rate. The two cultures chosen were selected as the best candidates as outlined in Chapter I objectives that also provided enough available dried biomass to perform a growth study.

2.2 Treatment designation and description of diet

Table 18. Treatment designation and description of diet (as-fed).

Treatment	Description of diet
Reference diet[1]	25% fishmeal, 0% test ingredient (as-fed)
Platymonas sp. diets[2]	
P20%	20% fishmeal, 5% *Platymonas* sp. (as-fed)
P40%	15% fishmeal, 10% *Platymonas* sp. (as-fed)
P60%	10% fishmeal, 15% *Platymonas* sp. (as-fed)
P80%	5% fishmeal, 20% *Platymonas* sp. (as-fed)
P100%	0% fishmeal, 25% *Platymonas* sp. (as-fed)
Spring Mix 2014 diets[2]	
SM20%	20% fishmeal, 5% Spring mix 2014 (as-fed)
SM40%	15% fishmeal, 10% Spring mix 2014 (as-fed)
SM60%	10% fishmeal, 15% Spring mix 2014 (as-fed)
SM80%	5% fishmeal, 20% Spring mix 2014 (as-fed)
SM100%	0% fishmeal, 25% Spring mix 2014 (as-fed)

[1]Reference diet contained 25% fishmeal
[2]Reference diets with various graded levels of test ingredient

2.3 Feed ingredient composition

The ingredient composition of the reference, *Platymonas* sp., and SM diets are shown in Tables 19 and 20. All diets were formulated to meet or exceed the requirements of *Oreochromis mossambicus* (NRC, 2011) using Concept 5^{TM} Formulation software (CFC Tech Services, Inc., Pierz, MN, USA). Casein was adjusted to maintain similar levels of crude protein as required for juvenile tilapia (NRC, 2011).

Table 19. Ingredient composition (g/kg dry wt.) of reference diet and five levels of *Playmonas* sp. diet (as-fed).

Ingredients	Reference	P20%	P40%	P60%	P80%	P100%
Casein[1]	6.10	6.94	7.78	8.62	9.46	10.30
Menhaden Fishmeal (62%)[2]	25.00	20.00	15.00	10.00	5.00	0.00
Soybean Meal[3]	35.00	35.00	35.00	35.00	35.00	35.00
DL-Met[4]	0.00	0.03	0.06	0.09	0.12	0.15
Vitamin Premix[5]	0.20	0.20	0.20	0.20	0.20	0.20
Mineral Premix[6]	0.30	0.30	0.30	0.30	0.30	0.30
Stay C[7]	0.50	0.50	0.50	0.50	0.50	0.50
Alginate[8]	2.00	2.00	2.00	2.00	2.00	2.00
Cellulose[9]	2.59	2.62	2.65	2.69	2.72	2.75
Krill Meal[10]	2.00	2.00	2.00	2.00	2.00	2.00
Menhaden Fish Oil[11]	2.00	2.00	2.00	2.00	2.00	2.00
$CaH_4P_2O_8$[12]	5.56	5.31	5.05	4.80	4.55	3.89
Wheat Starch[13]	13.96	13.33	12.70	12.08	11.45	11.23
Corn oil[14]	4.80	4.78	4.75	4.73	4.71	4.68
Platymonas sp.	0.00	5.00	10.00	15.00	20.00	25.00

[1] Sigma Aldrich, St. Louis, MO, USA
[2] Zeigler Bros. Inc., Gardners, PA, USA
[3] Producers Cooperative Association, Bryan, TX, USA
[4] Alfa Aesar, Reston, VA, USA
[5] Zeigler Bros. Inc., Gardners, PA, USA
[6] Zeigler Bros. Inc., Gardners, PA, USA
[7] Zeigler Bros. Inc., Gardners, PA, USA
[8] Acros Organics, NJ, USA
[9] Alfa Aesar, Reston, VA, USA
[10] Zeigler Bros. Inc., Gardners, PA, USA
[11] Zeigler Bros. Inc., Gardners, PA, USA
[12] PCS Sales, Saskatoon, Saskatchewan, Canada
[13] MP Biomedicals, Solon, OH, USA
[14] ACH food companies, Cordola, TN, USA

Table 20. Ingredient composition (g/kg dry wt.) of a reference diet and five levels of Spring mix 2014 diets (as-fed).

Ingredients	Reference	SM20%	SM40%	SM60%	SM80%	SM100%
Casein[1]	6.10	6.62	7.14	7.67	8.82	8.71
Menhaden Fishmeal (62%)[2]	25.00	20.00	15.00	10.00	5.00	0.00
Soybean Meal[3]	35.00	35.00	35.00	35.00	35.00	35.00
DL-Met[4]	0.00	0.03	0.06	0.10	0.13	0.16
Vitamin Premix[5]	0.20	0.20	0.20	0.20	0.20	0.20
Mineral Premix[6]	0.30	0.30	0.30	0.30	0.30	0.30
Stay-C[7]	0.50	0.50	0.50	0.50	0.50	0.50
Alginate[8]	2.00	2.00	2.00	2.00	2.00	2.00
Cellulose[9]	2.59	2.62	2.65	2.69	2.72	2.76
Krill Meal[10]	2.00	2.00	2.00	2.00	2.00	2.00
Menhaden Fish Oil[11]	2.00	2.00	2.00	2.00	2.00	2.00
$CaH_4P_2O_8$[12]	5.56	6.52	7.48	8.44	9.40	10.36
Wheat Starch[13]	13.96	12.76	11.57	10.38	8.55	7.99
Corn oil[14]	4.80	4.45	4.09	3.74	3.38	3.03
Spring mix 2014[15]	0.00	5.00	10.00	15.00	20.00	25.00

[1] Sigma Aldrich, St. Louis, MO, USA
[2] Zeigler Bros. Inc., Gardners, PA, USA
[3] Producers Cooperative Association, Bryan, TX, USA
[4] Alfa Aesar, Reston, VA, USA
[5] Zeigler Bros. Inc., Gardners, PA, USA
[6] Zeigler Bros. Inc., Gardners, PA, USA
[7] Zeigler Bros. Inc., Gardners, PA, USA
[8] Acros Organics, NJ, USA
[9] Alfa Aesar, Reston, VA, USA
[10] Zeigler Bros. Inc., Gardners, PA, USA
[11] Zeigler Bros. Inc., Gardners, PA, USA
[12] PCS Sales, Saskatoon, Saskatchewan, Canada
[13] MP Biomedicals, Solon, OH, USA
[14] ACH food companies, Cordola, TN, USA
[15]Spring mix 2014 – Outdoor culture at Texas A&M Agrilife. Spring 2014. *Microchloropsis salina*, *P. tricornutum* sp., and *Amphora* sp.

2.4 Nutrient composition of test diets

Proximate analysis of the experimental diets used standard methods described in the AOAC (1990). Moisture content was determined by oven drying at 105ºC until a constant weight was obtained; ash was quantified as the residual weight after combustion in a muffle furnace (Thermo Fisher Scientific, Richardson, TX, USA) at 550ºC overnight. The Dumas method

(Ebeling, 1968) was used to determine crude protein (N × 6.25) and amino acids were evaluated using the HPLC method (AOAC, 1990) Crude lipid was determined via chloroform and methanol extraction (Folch et al., 1957). The gross energy content of algae was measured by combustion in a bomb calorimeter (Parr Instrument Company, Moline, IL, USA) using a benzoic acid standard (Schlosser et al., 2005).

Nutrient level for experimental diets is shown in Appendices 1, 2, and 3 were formulated to meet or exceed recommended nutritional requirements for growth and survival of Mozambique tilapia (NRC, 2011). Protein, fat, and fiber values were fixed to achieve daily requirements (Table 20). Nutrient values of algal ingredients can be located in Chapter II (Table 10).

There were no significant differences in the nutritional composition in any of the experimental or reference diets (Table 21). The P20 diet had the highest amount of Met compared to all other P diets. The reference diet contained more Arg (2.46), Cys (0.42), Leu (3.15), Lys (2.91), Phe (1.85), and Thr (1.77) (Table 22) than any of the P diets. The reference diet had the highest amount of Arg (2.46), Cys (0.42), His (1.12), Leu (3.15), Lys (2.91), Phe (1.85), and Thr (1.77) compared to all other SM diets. The Met values (% crude protein) across all diets ranged from 0.79 (reference diet) to 0.98 (SM20 diet) (Table 23). All diets met or exceeded established EAA requirements for the growth and survival of *Oreochromis* spp.

Table 21. Analyzed composition values of experimental feeds (as-fed).

Diets	Dry Matter (%)	Ash (%)	Protein (%)	Lipid (%)	Energy (kcal/kg)
Reference	96.20	14.56	42.92	10.54	4559.88
P20%	95.62	13.86	42.05	11.12	4587.35
P40%	94.64	13.38	41.87	11.29	4630.58
P60%	94.46	13.86	41.10	11.83	4640.31
P80%	96.14	16.60	40.19	11.04	4381.01
P100%	94.16	16.01	40.68	12.28	4502.09
SM20%	95.46	15.68	41.49	10.99	4605.61
SM40%	95.56	15.53	41.30	11.18	4604.71
SM60%	95.72	15.11	41.32	11.48	4575.82
SM80%	95.29	14.53	41.48	11.19	4586.54
SM100%	94.53	14.71	40.60	11.15	4607.38

Table 22. Amino acid composition of *Platymonas* sp. ingredient diets.

Amino Acids*	Reference	P20%	P40%	P60%	P80%	P100%
Arg[1]	2.46	2.30	2.34	2.28	1.97	1.82
Cys[2]	0.42	0.44	0.45	0.43	0.38	0.33
His[1]	1.12	1.01	0.97	1.03	0.90	0.85
Ile[1]	1.73	1.67	1.63	1.72	1.61	1.67
Leu[1]	3.15	3.11	3.05	3.10	3.02	2.94
Lys[1]	2.91	2.80	2.67	2.63	2.52	2.35
Met[1]	0.79	0.88	0.78	0.82	0.91	0.78
Phe[1]	1.85	1.82	1.82	1.88	1.81	1.75
Thr[1]	1.77	1.74	1.61	1.72	1.62	1.62
Val[1]	1.99	1.96	1.89	1.99	1.91	1.99

[1] Essential amino acid

[2] Non-essential amino acid

*Analyzed by New Jersey Feed Labs, Ewing Township, NJ, USA (n=1)

Table 23. Amino acid composition of Spring mix 2014 diets.

Amino Acids*	Reference	SM20%	SM40%	SM60%	SM80%	SM100%
Arg[1]	2.46	2.27	2.21	2.10	1.97	1.84
Cys[2]	0.42	0.40	0.38	0.39	0.36	0.36
His[1]	1.12	0.99	1.04	0.93	0.95	0.85
Ile[1]	1.73	1.75	1.80	1.73	1.72	1.62
Leu[1]	3.15	3.05	3.11	3.04	3.11	2.87
Lys[1]	2.91	2.77	2.76	2.57	2.56	2.29
Met[1]	0.79	0.96	0.97	0.95	0.98	0.89
Phe[1]	1.85	1.77	1.78	1.75	1.79	1.65
Thr[1]	1.77	1.71	1.73	1.69	1.71	1.59
Val[1]	1.99	2.05	2.12	2.06	2.07	1.95

[1] Essential amino acid
[2] Non-essential amino acid
*Analyzed by New Jersey Feed Labs, Ewing Township, NJ, USA (n=1)

2.5 Source of fish and acclimation protocol

Mixed-sex juvenile tilapia (*Oreochromis mossambicus*) were obtained as ~13-mg juveniles from Green Springs Farm (Ashland, OR, USA) and allowed to acclimate to experimental conditions for one week at the Texas A&M AgriLife Mariculture Facility, Flour Bluff, TX, USA. During this time, the fish were fed the same reference diet used for the grow-out trial.

2.6 Stocking and experimental system

Upon achieving an initial mean weight of 170 ± 15 mg, juvenile tilapia were stocked into replicated 250-L treatment tanks (n = 5, water depth = 0.45 m, bottom area = 0.09 m^2) at a density of 10 fish per tank. The experimental system consisted of 55 tanks connected as a common recirculating aquaculture system. Tanks were covered with translucent plastic to prevent escape of fish and reduce evaporation. Water exchange was achieved by passing dechlorinated municipal water through a pressurized sand filter, biological filter, cartridge filter (100 µm), at a recirculating

rate of 0.5 L/min/tank or 3,470% per day, per tank. A photoperiod of 12L/12D was used. Each of the 11 dietary treatments was replicated among five randomly selected tanks.

2.7 Feed preparation

Dry ingredients used in the reference and experimental diets were first ground using a burr mill (Mr. Coffee Automatic Burr Mill, Neosho, MI, USA) and passed through a mesh sieve with a diameter of ~500 µm before being homogenized in a commercial food mixer for 30 minutes. Oils were added and continuously mixed for an additional 15 minutes using a Model A-200 mixer (Hobart Corporation, Troy, OH, USA). Alginate was added to warm deionized water (400 mL kg^{-1} diet) in a separate bowl and mixed using a hand mixer (Sunbeam Products Inc., Milford, MA) for approximately 45 seconds. The resulting gelatinous mixture including deionized water (300 mL kg^{-1}) were added to the ingredients and mixed for another 15 minutes to obtain the appropriate consistency for mash extrusion. A meat chopper attachment (Model A-800 Hobart #12, Hobart Corporation, Troy, OH) fitted with a 3-mm die was used for extrusion. Moist feed strands were dried on wire racks in a forced air oven at 40°C to ~8-10% moisture. Feed was milled to the appropriate size for fish consumption, sifted, bagged, and stored at 4°C.

2.8 Experimental protocol/system management

Experimental feeds were distributed on an individual tank basis eight times per day at even intervals by means of a Fish Mate F14 automated feeder (Petmate, Arlington, TX, USA). Uneaten feed and feces were removed daily before the next ration was distributed to each tank in the recirculating system. Water temperature, salinity, and dissolved oxygen were measured daily in a random tank within each block with a YSI 85 oxygen/conductivity instrument (YSI, Yellow

Springs, Ohio, USA). Total ammonia nitrogen, nitrite and nitrate levels of system water were determined weekly using a Hach DR/2100 spectrophotometer (Hach, Loveland, Colorado, USA).

2.9 Performance Metrics

The following equations were used to calculate various diet performance indices used in the present study:

1) Survival (%) = ((final number of fish/tank) × (100))/initial number of fish per tank

2) Weight gain (%) = ((final weight of fish per tank-initial weight of fish/tank) × (100))/initial weight of fish tank

3) Specific growth rate (SGR) = (final body weight (g) – initial body weight (g))/ (days of experimental trial^{-1}×100)

4) Feed conversion ratio (FCR)

= total weight of feed offered (g) per tank/weight weight gain (g)

5) Protein efficiency ratio (PER) = Weight gain (g)/ protein fed (g)

2.10 Statistical analyses

All data were subjected to a one-way analysis of variance (ANOVA $\alpha = 0.05$) using R (5.3.1; Feather Spray, The R Foundation) statistical package for Windows and presented as means ± SD. Independent variables included test ingredient and level of fishmeal replacement. Data were

analyzed as a randomized block design with five levels of *Platymonas* sp., five levels of Spring mix 2014, and a reference diet constituting one "block". All dependent variables were tested for normality before statistical analysis and included: initial weight, final body weight, weight gain (%), survival (%), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER). Water quality factors were reported as minimum, maximum, and means ± standard deviation.

3. Results

3.1 Water quality

All water quality variables were within appropriate range for the growth and survival of *O. mossambicus* (Table 24).

Table 24. Mean values for water quality variables over trial period (n=4).

Variable	Mean ± SD
Temperature (^oC)	28.4 ± 0.47
Salinity (ppt)	1.02 ± 0.37
pH	8.27 ± 0.17
D.O. (mg/L)	8.26 ± 0.46
NH_4 (mg/L)	0.09 ± 0.04
NO_3 (mg/L)	0.08 ± 0.16
NO_2 (mg/L)	1.96 ± 0.82

3.2 Survival and growth indices

Survival of juvenile tilapia fed the *Platymonas* sp.-based diet were statistically similar ($P \geq 0.05$) and ranged from 96-100% (Table 25). Percent weight gain ranged from 2,565 – 2,991% over the 30-day feeding trial and was significantly lower ($P = 0.0064$) in the diet in which all FM had been replaced (P100%). Specific growth rate was similar ($P = 0.5674$) for all diets and ranged from 11.34 – 10.91, P80% and P100%, respectively. Feed conversion ratio ranged from 1.33 ±

0.01 – 1.19 ± 0.04 (P40% and P100%, respectively). Final weight of tilapia ranged from 46.95 ± 0.30 (P40%) – 39.44 ± 5.16 g with those fed the P100% diet being significantly lower (P = 0.0062). Protein efficiency ratio of juvenile tilapia fed diets at different levels of FM replacement with the *Platymonas* sp.-based diets ranged from 3.32 (P40%) – 3.43 (P20%). The PER was significantly higher (P =0.0214) for those fish fed P20%, P40%, and P80% diets vs. the P100% diet.

Tilapia fed diets in which graded levels of FM was replaced by Spring Mix 2014 all had 100% survival (Table 26). Percent weight gain ranged from 2,842 – 3,028% with no significant difference among treatments (P = 0.1013). Specific growth rate ranged from 11.26 ± 0.37 – 11.57 ± 0.18, with no significant difference in dietary response (P = 0.4022). Final weight gain was similar (P = 0.1054) for all dietary treatments and ranged from 43.43 ± 3.52g - 48.34 ± 1.49g. Feed conversion ratio was similar (P = 0.1139) for all treatments and ranged from 1.28 ± 0.02 – 1.33 ± 0.02. Protein efficiency ratio ranged from 3.22 ± 0.53 – 3.42 ± 0.12 and was not significantly different from any other SM dietary treatment (Table 26).

Table 25. Final mean weight, weight gain, survival, specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) of *O. mossambicus* fed graded levels of *Platymonas* sp. in 30-day trial. Values are expressed as the mean (± SD) of five replicate tanks, on a wet weight basis. Values with different superscripts in the same column indicate significant differences determined by Tukey's test ($P < 0.05$).

Diets	Survival (%)	Wt. gain (%)	SGR	Final Wt. (g)	FCR	PER
Reference	100	2,842.21 ± 362.8^{a}	11.28 ± 0.37^{a}	45.31 ± 2.91^{a}	1.28 ± 0.05^{ab}	3.37 ± 0.07^{ab}
P20%	100	2,865.61 ± 284.6^{a}	11.31 ± 0.33^{a}	46.56 ± 1.82^{a}	1.30 ± 0.03^{a}	3.43 ± 0.15^{a}
P40%	96	2,728.31 ± 378.8^{a}	11.18 ± 0.48^{a}	46.95 ± 0.30^{a}	1.33 ± 0.01^{a}	3.32 ± 0.35^{a}
P60%	98	2,830.46 ± 285.1^{a}	11.27 ± 0.31^{a}	44.25 ± 1.22^{a}	1.28 ± 0.02^{ab}	3.39 ± 0.22^{ab}
P80%	100	2,990.54 ± 372.9^{a}	11.34 ± 0.25^{a}	45.74 ± 1.47^{a}	1.28 ± 0.02^{ab}	3.33 ± 0.13^{a}
P100%	100	2,564.86 ± 344.3^{b}	10.91 ± 0.36^{a}	39.44 ± 5.16^{b}	1.19 ± 0.14^{b}	3.37 ± 0.35^{b}
P value	-	0.0064	0.5674	0.0062	0.0145	0.0214

Table 26. Final mean weight, weight gain (%), survival, specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) of *O. mossambicus* fed graded levels of Spring Mix 2014 in 30-day trial. Values are expressed as a mean (± SD) of five replicate tanks, wet weight basis.

Diets	Survival (%)	Wt. gain (%)	SGR	Final Wt. (g)	FCR	PER
Reference	100	2,842.21 ± 362.8	11.28 ± 0.37	45.31 ± 2.91	1.28 ± 0.05	3.37 ± 0.07
SM20%	100	3,167.33 ± 279.3	11.57 ± 0.18	48.28 ± 1.32	1.44 ± 0.01	3.41 ± 0.11
SM40%	100	2,849.69 ± 339.2	11.26 ± 0.37	48.34 ± 1.49	1.33 ± 0.02	3.42 ± 0.12
SM60%	100	3,027.89 ± 325.3	11.56 ± 0.19	46.52 ± 2.35	1.30 ± 0.04	3.33 ± 0.20
SM80%	100	2,879.73 ± 179.6	11.18 ± 0.51	45.50 ± 2.28	1.30 ± 0.07	3.34 ± 0.25
SM100%	100	2,898.70 ± 342.5	11.48 ± 0.25	43.43 ± 3.52	1.28 ± 0.02	3.22 ± 0.53
P value	-	0.1031	0.4022	0.1054	0.1139	0.0685

4. Discussion

Most feeding trials using *Platymonas* sp. have focused on its use as a source of nutrition for filter-feeding organisms such as *Artemia* sp. (Li et al., 2008), copepods (Yu et al., 2015), and bivalves (Wang et al., 2016). *Platymonas helgolandica* has been used as a water quality amendment in culture of *Litopenaeus vannamei* to improve resistance to *Vibrio parahaemolyticus*, improving growth, final weight, and survival as well as reducing levels of ammonia, nitrite, and nitrate in the water column. In vertebrates, a single study on 10-day old flounder larvae (~4 mm) utilizing *Platymonas* sp. showed that the larvae got little to no nourishment from *Platymonas* sp. (Orcutt, 1950). More recently, the use of *Platymonas* sp. was examined in filter-feeding silver carp which showed that the *Platymonas* cell was too small to be filtered and used for nutrients (Ma et al., 2010). There are currently no studies on the nutritional value of *Platymonas* spp. in larger vertebrates. However, it contains a crude protein content greater than that of SBM, has potential for good productivity under the right growth conditions, and is able to grow on inexpensive media. Therefore, *Platymonas* sp. was chosen for further evaluation for this study.

Results from feeding trials to evaluate replacement of fishmeal with dried *Platymonas* sp. biomass in semi-commercial production diets fed to juvenile tilapia indicated both high survival and comparable growth response at high levels of fishmeal replacement. Fish offered the *Platymonas* sp. diets showed a significant decrease in terms of percentage weight gain at the 100% replacement level. This indicates that biomass from *Platymonas* sp. can effectively replace up to 80% of fishmeal in semi-commercial production diets for tilapia without sacrificing growth or survival.

The Spring mix 2014 dried biomass (*Microchloropsis salina, P. tricornutum,* and *Amphora* sp.) was used for two independent studies in 2016 and 2017. DeCruz et al. (2018) used the mixture

to evaluate the effect of fishmeal replacement in the diet of hybrid striped bass. In this work, they were able to substitute up to 15% crude protein in the formulated diet without sacrificing growth or survival. In this study, up to 100% of fishmeal could be replaced in the diet of *O. mossambicus* with no effect on growth and survival. Although only two studies have been performed on this mix specifically, dozens of studies have been performed utilizing *Microchloropsis* spp. and *P. tricornutum* as a potential feed replacement in aquaculture diets. Many of these studies have been performed on invertebrates such as; Pacific oyster (Brown et al., 1998), rotifers (Chebil et al., 1998), artemia (Zhukova, et al., 1998), and larval fishes such as *Paralichthys olivaceus* (Furuita et al., 1998) and *Sparus aurata* (Navarro et al., 1998). Gabadamosi and Lupatsch (2018) supplemented soybean meal with *M. salina* meal completely as feed for juvenile Nile tilapia at a level of 35% crude protein. The trial showed that the fish had better FCR and PER when fed with *M. salina* versus a soybean-based diet.

Additionally, *P. tricornutum* has been extensively evaluated due to its potential to produce DHA (C22:6n-3) and EPA (C20:5n-3) (Atalah, et al., 2007; Ibanez et al., 1998; Sorensen et al., 2016; Qiao et al., 2016). Changes in culture conditions can affect this ratio (Qiao et al., 2016). Many of these studies were performed in media such as f/2 or LDM which can be prohibitively expensive for commercial use (Huysman et al., 2015; Qiao et al., 2016; Vazhappilly and Chen, 1998). Although a larger body of research is available on the use of *P. tricornutum* in aquaculture diets, many of those are also nutritional studies for its use in invertebrate nutrition. In one study, Atlantic salmon were offered a diet of *P. tricornutum* (Sorensen et al., 2016). It was found that that dried biomass of *P. tricornutum* could be used to replace up to 6% of diet without adverse effects on digestibility, FCR, or growth. In this study *P. tricornutum* was cultured outdoors, in ODI media to determine its potential as an aquaculture feed. Unfortunately, *P. tricornutum* did

poorly and produced the lowest biomass (Chapter II) by comparison to the other algae. This was potentially due to the weather patterns of south Texas as it has been cultured successfully in the same location in a previous study under different climatological conditions (Huysman et al., 2015).

Amphora sp. has been studied as a supplemental food item for grazing by planktivorous *Oreochromis* spp. (Norberg et al., 2008) and *Sartherodon melanotheron* (Tidiani et al., 2003). However, neither of these studies utilized *Amphora* sp. as a dried feed ingredient. As with *Platymonas* sp., *Amphora* sp. is primarily fed as live cells in the water for invertebrates such as *L. vannamei* (Martins et al., 2014), *Mithraculous forceps* (Penha-Lopes et al., 2006), and *Haliotidae* sp. (Yuyu et al., 2010).

Although *M. salina, P. tricornutum* and *Amphora* sp. were not used individually in the above referenced growth trial, it is highly recommended that research continues into the use of these species for fishmeal replacement in aquaculture. Based on our findings, it was expected that a diet composed of these species would serve as an appropriate partial substitution for fishmeal. This research shows that up to 100% of fishmeal can be replaced with a mixed diet of these three species with no significant differences on final weight, weight gain (%), survival (%), specific growth rate (SGR), feed conversion ratio (FCR), or protein efficiency ratio (PER) of *O. mossambicus*.

Tilapia are currently the second most farmed fish in the world and although their diets can already be produced utilizing alternative sources to fishmeal (e.g. soybean meal) without sacrificing growth or survival. However, the use of soybean meal in tilapia diets increases dependence on freshwater resources decreasing the supply available for human use. Therefore, *Microchloropsis* spp. and *P. tricornutum* specifically need futher study in the diets of *Oreochromis* spp., as these algae are becoming more increasingly used for lipid extraction to produce biodiesel

(Converti et al., 2009; Moazami et al., 2012; Umdu et al., 2009) and reduces the use of freshwater needed to produce algal biomass.

Marine algae, with high growth rate, as well as a high protein and lipid content, that can be cultured in inexpensive media have the potential to be beneficial to both the biofuel and feed industry. Marine algae are considered to be the most important renewable resource in the production of biodiesels (Demirbas, 2010) and are the subject of frequent evaluation to determine their usefulness for biofuel production (Beal et al., 2015; Demirbas, 2010; Sills et al., 2014).

Unfortunately, the cost of production of algae biomass remains high. Many of the costs are associated with harvesting, dewatering, and lipid extraction. However, the costs may be slightly offset by reducing the cost of the nutrient media necessary to produce biomass. Additionally, in order to make the remaining biomass viable for use as an aquaculture feed, there are numerous criteria that must be further considered; 1) the location of the farms must be suitable for the growth selected species year-round, 2) the species chosen must outcompete naturally occurring organisms that could serve as contaminants, 3) algal cultures must be harvested throughout different periods of the year so that production of biomass can be maintained, 4) nutrient levels in biomass must remain consistent for shipment to feed producers, 5) non-chemical lipid separation techniques must be improved, and 6) algae identified as a potential feed additive should undergo lipid extraction and reevaluated as a potential fishmeal replacement in tilapia diets. Therefore, extensive research and development is still required to make the use of algae as a fishmeal replacement in aquaculture feeds a feasible alternative.

CHAPTER V: CONCLUSIONS

In this study, two dozen species of algae were analyzed as potential replacements or substitutes for fishmeal in production diets fed to tilapia. Individual *Microchloropsis salina, Amphora* sp., *Platymonas* sp., *Cylindrotheca* sp., and a mix of *M. salina, P. tricornutum,* and *Amphora* sp., as well as a mixture of *M. salina, P. tricornutum*, and *Cylindrotheca* sp., achieved levels of productivity outdoors to provide large amounts of biomass needed for use by feed manufacturers. All outdoor cultures examined were able to produce biomass on a low-cost nutrient medium and all contained levels of crude protein in excess of de-hulled soybean meal (~48.5%) (NRC, 2011). All of the cultures tested could potentially be used in feed formulations, but none of the cultures exhibited a higher crude protein digestibility than has been reported for most fish meals (Fontainhas-fernandes, et al., 1999; Koprucu and Ozdemir, 2005; Popma, 1982).

Outdoor productivity trials with *Cylindrotheca* sp. indicated good potential for providing sufficient production in different growout conditions. Inclusion of *Cylindrotheca* sp. as a major ingredient warrants further evaluation for use in *O. mossambicus* diets as little research has been performed on *Cylindrotheca* spp. as a component of aquaculture diets. Although growth and survival have been examined in abalone (Matsumoto et al., 2015, 2018), and sea cucumbers (Junwei et al., 2015) fed *Cylindrotheca closterium*, no other research exists evaluating *Cylindrotheca* spp. formulated into the diet of aquatic vertebrates as as a dry feed ingredient. In this study, the diatoms exhibited low dry matter digestibility (57.9 ± 3.5%) in a diet containing 30% of this strain of algae (Chapter III). Other research has shown a trend of decreased digestibility of a diet with increased dietary levels of another diatom, *P. tricornutum* (Sorensen et al., 2016). Sorenson et al., (2016) found that the *P. tricornutum* could be supplemented in the diet to replace

only 6% of fishmeal without adverse effects on digestibility, FCR, and growth. Additionally, the culture of certain diatoms (such as *Cylindrotheca* sp.) presents a need for silica supplementation in the nutrient media that will further increase the costs associated with microalgae production. Cyanobacteria have been shown to improve saturated fatty acids and collagen content (Liang et al., 2015). Additionally, *Arthrospira platensis* (*Spirulina*), has been shown to enhance growth performance of tilapia when up to 30% of the diet was replaced (Velasquez et al., 2016). *Spirulina* and may also offer the added benefit of tissue protection and serve as antioxidants in *O. niloticus* (Ibrahema and Ibrahim, 2014). These health benefits may be due to high levels of digestibility (Sarker et al., 2015). Unfortunately, the use of certain cyanobacteria in aquaculture feeds have also been shown to increase toxic microcystin content in muscle tissues in *Carassius auratus* (Liang et al., 2015), *Cyprinus carpio* (Li et al., 2014), and *Oreochromis niloticus* (Palikova et al., 2011). Additionally, *Oreochromis mossambicus* fry have been shown to reject feed when fishmeal is replaced by *Spirulina* at ≥ 60% (Olvera-Novoa et al., 1998). In this study, the cyanobacterial diet was also consumed at a very low rate compared to the other test diets. Therefore, enough feces were not produced for evaluation. For this reason, it was eliminated from further consideration in both the digestibility and the growth trial. It is concluded that future research might focus on addressing on the potential toxins as well as the palatability of cyanobacteria in the diet of *O. mossambicus* to determine its utility in future studies.

Based upon the digestibility results, *Platymonas* sp. was evaluated for replacement of fishmeal in growth trials with tilapia (Chapter IV). Results indicated that, for this particular ingredient sample, a diet composed of 100% *Platymonas* sp. in replacement of fishmeal (FM) reduced growth and feed performance in *O. mossambicus* cultures. The *Platymonas* sp. (P) diet that replaced 100% of fishmeal had a lower final weight (g), weight gain (%) and specific growth

rate (SGR) in relation to all other diets. However, the 100% substitution diet also resulted in the lowest feed conversion ratio (1.19 ± 0.14). These results could not be directly compared with other studies as the genera is known for use in Artemia (Li et al., 2008), copepods (Yu et al., 2015), and bivalves (Wang et al., 2016; Yang et al, 2013; Yuewen et al., 2013), and no studies were found using *Platymonas* sp. in formulated fish diets.

In contrast to the *Platymonas* sp. diets, all inclusion levels of the spring mix 2014 (SM) and the reference diet showed no significant difference in any performance metric. This suggests that inclusion levels up to 100% SM in replacement of fishmeal did not affect any of the measured metrics (Chapter IV). Studies have been performed utilizing both *M. salina, P. tricornutum,* and *Amphora* sp. substituted for fishmeal in formulated feeds at various levels. DeCruz et al. (2018) were able to substitute up to 15% crude protein in the diets of hybrid striped bass without negatively effecting growth or survival. In 2018, Gabadamosi and Lupatsch (2018) used *M. salina* to completely replace soybean meal at a level of 35% crude protein in Nile tilapia diets. The trial showed that the fish had better FCR and PER when fed *M. salina* versus a soybean-based diet. Based on the findings of the above referenced research it is not surprising that certain levels of the spring mixture would serve well as a partial fishmeal replacement. However, it was not expected that there would be no significant differences in the performance metrics between all diets. Therefore, further evaluation is necessary to determine economic sustainability of this algal mixture as a fishmeal replacement as no levels of the SM treatment compromised growth (%), survival (%), SGR, FCR, or PER.

This study shows that the acceptable range of inclusion levels possible as a partial fishmeal replacement is broad in the *Platymonas* sp. diet (0-80%) and may make fishmeal supplementation feasible in diets with cultures containing these species. However, the spring mix 2014 requires

further evaluation. Although there were no significant differences in performance between any of the spring mix or the reference diets, studies have shown conflicting evidence in the use of diatoms in aquaculture diets such as the Sorensen et al., 2016 study referenced above. These results, as well as those in this study, suggest that mixtures such as the Spring mix 2014 as a fishmeal replacement may be dependent upon the ratios of the alga at harvest.

Although algae are widely used in hatcheries, a limited amount of research has been performed using algae as a fishmeal replacement in formulated diets of vertebrates such as *O. mossambicus*. Thus, it is important to determine why these cultures and their processing method were able to replace fishmeal in the diet of tilapia at high percentages (80% *Platymonas* sp. and 100% spring mix 2014). The above referenced factors in the body of this dissertation show that an increased understanding of the nutritional benefits of the algae and their mixes used in this research need closer examination to include the lipid profiles of the algae, and of tilapia muscle tissue. There is need for standardization of cultivation as nutritional properties change due to different biotic and abiotic factors affecting the algae. Therefore, it is imperative that nutritive value under various culture conditions be evaluated. The properties of the algae need to be investigated after processing to determine the digestibility and bioavailability of the ingredients as protein solubility varies between different species of algae (Teuling et al., 2017) and the understanding of the individual constituents are almost unknown. This research is unique as it is the first performed on these particular algae as a fishmeal replacement in formulated feeds for *O. mossambicus* fry (starting weight ~0.170mg). It has been shown that tilapia fry, with supplemental feeding, still obtain up to 50% of their growth from natural organisms, such as algae. In contrast, channel catfish will only obtain 10% of their growth under the same conditions (Popma and Masser, 1999). It is clear that *Oreochromis mossambicus* have the ability to effectively utilize algal biomass for

growth. The two cultures that were used in the diets for the growth trial (*Platymonas* sp., and Spring mix 2014) showed strong potential for fishmeal replacement in tilapia diets (80% and 100%, respectively). A salt water algal species, with high protein and lipid content, that effectively utilizes inexpensive media for daily biomass production, has the potential to be beneficial to both the biofuel and feed industry without competing for necessary human resources (e.g. water or soybean meal).

Unfortunately, the cost of algae biomass production is still quite high. Many of the costs are associated with harvesting, dewatering, and lipid extraction. However, the costs may be slightly offset by reducing the cost of the nutrient media necessary to produce biomass. Additionally, in order to make the remaining biomass viable for use as an aquaculture feed, there are numerous criteria that must be further considered; 1) the location of the farms must be suitable for the growth selected species year-round, 2) the species chosen must outcompete naturally occurring organisms that could serve as contaminants, 3) algal cultures must be harvested throughout different periods of the year so that production of biomass can be maintained, 4) nutrient levels in biomass must remain consistent for shipment to feed producers, 5) non-chemical lipid separation techniques must be improved, and 6) algae identified as a potential feed additive should undergo lipid extraction and reevaluated as a potential fishmeal replacement in tilapia diets. Therefore, extensive research and development is still required to make the use of algae as a fishmeal replacement in aquaculture feeds a feasible alternative.

CHAPTER VI: REFERENCES

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APPENDICES

Appendix 1. Formulated nutrient levels in reference diet (as-fed basis).

Nutrient	Units	Level	Nutrient	Units	Level
Dry Matter	PCT	88.07	Val	PCT	2.14
Moisture	PCT	11.93	Leu	PCT	3.10
Protein	PCT	40.00	Arg	PCT	2.46
Fat	PCT	10.00	Phe	PCT	1.93
Fiber	PCT	3.90	Phe + Tyr	PCT	3.25
Ca	PCT	2.62	Na	PCT	0.32
Phos total	PCT	2.04	P	PCT	0.85
Ash	PCT	13.00	Mg	PCT	0.17
Phos avail	PCT	0.78	S	PCT	0.15
Carb	PCT	16.09	Mn	PPM	44.35
Marine Lip	PCT	4.64	Fe	PPM	238.10
Total energy	Kcal/g	789.55	Cu	PPM	37.58
Met	PCT	0.90	Zn	PPM	147.37
Cys	PCT	0.43	Se	PPM	0.63
Lys	PCT	2.81	Cobalt	PPM	0.76
Trp	PCT	0.49	Iodine	PPM	0.88
Thr	PCT	1.66	Cholesterol	PPM	104.35
Iso	PCT	1.90	Choline	PPM	2131.95
His	PCT	1.11	Inositol	PPM	589.00

Appendix 2. Nutrient values of formulated experimental diets (as-fed basis) for evaluation of *Platymonas* sp. as a fishmeal replacement in *O. mossambicus* diets.

Nutrient	Unit	P20%	P40%	P60%	P80%	P100%
Dry Matter	PCT	88.08	88.09	88.10	88.10	87.98
Moisture	PCT	11.92	11.91	11.90	11.90	12.02
Protein	PCT	40.00	40.00	40.00	40.00	40.00
Fat	PCT	10.00	10.00	10.00	10.00	10.00
Fiber	PCT	3.90	3.90	3.90	3.90	3.90
Ca	PCT	2.32	2.01	1.70	1.39	1.00
Phos total	PCT	1.86	1.67	1.49	1.31	1.06
Ash	PCT	13.00	13.00	13.00	13.00	12.61
Phos avail	PCT	0.72	0.65	0.59	0.52	0.45
Carb	PCT	15.41	14.73	14.04	13.36	13.09
Marine Lip	PCT	4.16	3.68	3.20	2.72	2.24
Total energy	Kcal/ g	764.67	739.79	714.90	690.02	682.17
Met	PCT	0.90	0.90	0.90	0.90	0.90
Cys	PCT	0.41	0.39	0.37	0.36	0.34
Lys	PCT	2.76	2.70	2.65	2.60	2.55
Trp	PCT	0.47	0.44	0.42	0.40	0.37
Thr	PCT	1.66	1.66	1.66	1.66	1.66
Iso	PCT	1.88	1.85	1.83	1.80	1.77
His	PCT	1.08	1.06	1.03	1.01	0.98
Val	PCT	2.14	2.13	2.13	2.13	2.12
Leu	PCT	3.16	3.23	3.29	3.35	3.41
Arg	PCT	2.48	2.49	2.51	2.52	2.54
Phe	PCT	1.95	1.97	1.99	2.01	2.03
Phe + Tyr	PCT	3.10	2.96	2.81	2.66	2.52
Na	PCT	0.30	0.28	0.26	0.24	0.22
P	PCT	0.82	0.78	0.75	0.71	0.69
Mg	PCT	0.16	0.15	0.15	0.14	0.13
S	PCT	0.16	0.16	0.16	0.16	0.16
Mn	PPM	41.93	39.51	37.09	34.67	31.02
Fe	PPM	210.89	183.69	156.48	129.27	96.85
Cu	PPM	36.87	36.15	35.43	34.71	33.67
Zn	PPM	139.64	131.91	124.19	116.46	107.83
Se	PPM	0.52	0.41	0.30	0.19	0.08
Cobalt	PPM	0.72	0.68	0.64	0.60	0.51
Iodine	PPM	0.80	0.72	0.64	0.56	0.43
Cholesterol	PPM	104.32	104.30	104.27	104.25	104.22
Choline	PPM	1822.50	1513.04	1203.59	894.14	599.97
Inositol	PPM	482.40	375.80	269.20	162.60	100.00

Crude protein, fats, and fiber were fixed to meet or exceed minimum daily requirements for *O. mossambicus.*

Appendix 3. Nutrient values of formulated experimental diets (as-fed basis) for evaluation of Spring mix 2014 as a fishmeal replacement in *O. mossambicus* diets.

Nutrient	Unit	SM20%	SM40%	SM60%	SM80%	SM100%
Dry Matter	PCT	87.88	87.69	87.49	87.30	87.10
Moisture	PCT	12.12	12.32	12.51	12.70	12.90
Protein	PCT	40.00	40.00	40.00	40.00	40.00
Fat	PCT	10.00	10.00	10.00	10.00	10.00
Fiber	PCT	3.90	3.90	3.90	3.90	3.90
Ca	PCT	2.57	2.51	2.46	2.40	2.35
Phos total	PCT	2.07	2.10	2.14	2.17	2.21
Ash	PCT	13.00	13.00	13.00	13.00	13.00
Phos avail	PCT	0.72	0.65	0.58	0.51	0.44
Carb	PCT	14.84	13.59	12.35	11.10	9.85
Marine Lip	PCT	4.16	3.68	3.20	2.72	2.24
Total energy	Kcal/g	740.91	692.26	643.61	594.96	546.32
Met	PCT	0.90	0.90	0.90	0.90	0.90
Cys	PCT	0.47	0.51	0.55	0.59	0.63
Lys	PCT	2.91	3.01	3.11	3.20	3.30
Trp	PCT	0.46	0.44	0.41	0.38	0.36
Thr	PCT	1.64	1.62	1.60	1.57	1.55
Iso	PCT	1.89	1.87	1.87	1.83	1.82
His	PCT	1.08	1.04	1.01	0.98	0.95
Val	PCT	2.16	2.17	2.19	2.20	2.22
Leu	PCT	3.14	3.18	3.22	3.26	3.30
Arg	PCT	2.39	2.32	2.25	2.18	2.11
Phe	PCT	1.92	1.91	1.90	1.89	1.88
Phe + Tyr	PCT	3.07	2.90	2.72	2.54	2.36
Na	PCT	0.30	0.28	0.26	0.24	0.22
P	PCT	0.82	0.78	0.75	0.72	0.68
Mg	PCT	0.17	0.17	0.16	0.16	0.16
S	PCT	0.15	0.16	0.16	0.16	0.16
Mn	PPM	45.55	46.75	47.96	49.16	50.36
Fe	PPM	226.85	215.60	204.35	193.10	181.85
Cu	PPM	37.81	38.04	38.27	38.50	38.74
Zn	PPM	142.22	137.08	131.93	126.78	121.64
Se	PPM	0.53	0.43	0.32	0.22	0.12
Cobalt	PPM	0.88	0.99	1.11	1.23	1.35
Iodine	PPM	0.94	0.99	1.04	1.09	1.14
Cholesterol	PPM	104.32	104.30	104.27	104.25	104.22
Choline	PPM	482.40	375.80	269.20	162.60	56.00
Inositol	PPM	100.00	100.00	100.00	100.00	100.00

Crude protein, fats, and fiber fixed to meet or exceed minimum daily requirements for *O. mossambicus*.