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Feasibility of Using Biofuel By-Products as a Sustainable Nutritional Resource for Aquaculture Production of Litopenaeus vannamei

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FEASIBILITY OF USING BIOFUEL BY-PRODUCTS AS A SUSTAINABLE NUTRITIONAL RESOURCE FOR AQUACULTURE PRODUCTION OF *LITOPENAEUS VANNAMEI*

By

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ABSTRACT

FEASIBILITY OF USING BIOFUEL BY-PRODUCTS AS A SUSTAINABLE NUTRITIONAL RESOURCE FOR AQUACULTURE PRODUCTION OF *LITOPENAEUS VANNAMEI*

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Many different algal species can provide an acceptable protein ingredient, with good digestibility, for shrimp feeds. Compared to fish meal, similar protein, carbohydrate, and lipid levels can be found in select algal species. Traditional shrimp diets in aquaculture rely on fish meal and fish oil from pelagic fish fisheries. A reduction or elimination of these ingredients would reduce the dependency of shrimp aquaculture on offshore fisheries and increase economic competiveness. Biofuel production produces algal by-products of potential use to aquaculturists that might reduce or eliminate the need for fisheries products in shrimp feed. Established uses for by-products from biofuel production include fertilizer for crops, fodder for swine and poultry, and production of methane and alcohol fuels. However, using biofuel production by-products as a protein and carbohydrate source for the Pacific white shrimp, *Litopenaeus vannamei*, has not been investigated. Therefore, a series of feeding experiments were conducted to evaluate if the algae used to produce biofuel could be a suitable main protein source in formulated diets for *L. vannamei*.

The feasibility of substituting biofuel algae by-product for fish meal in the juvenile *L. vannamei* (0.0306 ± 0.0011 g) diet was evaluated, and an adequate substitution ratio was determined. Eighteen experimental diets were evaluated using 60, 80, and 100% fish meal substitution levels. *Chaetoceros calcitrans*, *Nannochloropsis salina*, and *Pavlova sp*. were chosen as the algae sources as they have potentially high use in

biodiesel production due to their high lipid content and each has been included in established larval shrimp aquaculture operations. Each diet varied the level of fish meal substitution (60, 80, or 100%) and either contained dried algal biomass or, alternatively, dried algal biomass with reduced lipid content to simulate algal biomass post-biodiesel production. The diets were compared, relative to their effect on weight gain in juvenile *L. vannamei*, to each other and to a commercially available diet (CONTROL) and a diet formulated using the ingredients used in all of the experimental diet formulations but without algal biomass (BASAL).

The shrimp were held individually in 355-ml Styrofoam cups filled with 200-ml seawater with a salinity of 32 parts per thousand (ppt) salinity under a 12:12 light:dark photoperiod. Water exchange was 90% per day for six days and 100% on the seventh day when weights were taken. Each of the twenty diets was presented daily to seven replicate cups, each cup containing a single shrimp, for six weeks. Food was presented once per day to satiation, which was determined by the shrimp refusing additional feed. Each animal was weighed weekly. After six weeks, the shrimp were harvested and final weights were taken.

The analysis of differences between strains, levels, and lipids indicated there was a significant difference between all of the algal-based diets and the control. Overall, significantly better growth rates were observed in the diets with less fish protein replacement. The 60% fish meal replaced diets outperformed the diets that had 80 or 100% fish meal replacement. There were no significant differences in nutritional value among the algal species. Survival rates, from an aquaculture perspective, were acceptable for all treatments (>71%).

Results from these studies demonstrated that formulated diets using algal biomass

from biodiesel production can be the primary protein source for *L. vannamei* postlarvae.

KEYWORDS: pacific white shrimp, nutrition, algae, protein, replacement diet, lipids, feasibility

DEDICATION

To my parents and my wife for their unconditional support throughout my education.

ACKNOWLEDGEMENTS

Many will say it is the journey and not the destination that is what makes lasting memories and impressions. For me, this journey began when I was younger and I spent summers in the Catskill Mountains of New York with my grandparents. My grandfather and I would spend hours catching anything and everything that I could run down, catch in a cup, or sweep up into a net. Taking my day's "haul" back to the house, we would look through Audubon and Smithsonian identification books and fill the pages of my field journal. For his time and my grandmother's patience, I am always grateful.

My parents, Frank and Elaine, for their constant support and encouragement throughout my academic career with words of wisdom along the way, I will always be indebted. My siblings, Michelle and Kirk, continue to show me that most of the great things in life take patience and persistence to achieve. To my wife, Janaina, and my wonderful daughters who have sacrificed sharing me with time in the laboratory and the library, you all are in my hearts.

I would never have been able to finish my dissertation without the guidance of my committee members. Foremost, I would like to express my gratitude to my advisor Dr. Richard Spieler for his guidance, patience, critical reviews, and support throughout this thesis. I also offer sincere thanks to my committee members for guiding my research and providing the support during this endeavor and my academic career. Dr. James Thomas for his friendly guidance, thought-provoking suggestions, and overall support over the years. Dr. Curtis Burney whose class I first took years ago when I started my masters and led me to appreciate marine chemistry. Very special thanks to Dr. Rolland Laramore who introduced me to the concepts and research methods in the field of crustacean nutrition and anatomy. Their generous contribution of time and knowledge helped make this endeavor possible.

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Introduction

Biofuels are defined as energy carriers produced from the conversion of lignocellulosic or cellulosic biomass to provide sustainable inputs for heat, power, and transport applications. Biofuels are included in a broad group of alternative fuels that are made from non-petrogenic sources as defined in the Energy Policy Act of 2005 (U.S. Congress, 2005). The term alternative fuel does include fossil-derived fuels such as liquefied petroleum gas and natural gas whereas biofuels are those that are made from only non-fossil sources.

Biofuels can be divided into two groups – low blend biofuels and high blend / pure alternative biofuels. Low blend biofuels are used to blend into base fuels and include biodiesel or fatty acid methyl ester (FAME), bioethanol, bio-ethyl tertiary butyl ether (ETBE, bioethanol 37% and fossil isobutylene 63%), methyl tertiary butyl ether (MTBE), and paraffinic biofuels (Kampman *et al*., 2013). High blend and pure alternative biofuels such as biomethane, E100, methanol and dimethyl ether (DME) have been developed as a complete replacement of fossil fuel (Kampman *et al*., 2013). Biodiesel is a fatty acid methyl ester fuel derived from vegetable oils, animal fats, or other waste oils (Solecki *et al*., 2013).

Starting in the late 1970s, the Aquatic Species Program (ASP) at the Solar Energy Research Institute (SERI) was initiated to investigate the ability of macroalgae, microalgae, and emergent plants for their ability to make lipids and carbohydrates (Sheenan *et al*., 1998). Lipids could be used as a feedstock for liquid fuel or chemical production and carbohydrates can be fermented into ethanol or anaerobic digestion for methane production (Sheenan *et al*., 1998). In the 1980s, the decision was made to focus research at ASP on microalgae due to the ability for microalgae to produce lipids as the primary storage molecule (Sheenan *et al*., 1998). Since then, the science of biofuel production has been developed and production continues to increase each year (Kampman *et al*., 2013). Established uses for the by-products from biofuel production include fertilizer for crops, fodder for swine and poultry, production of

methane and alcohol fuels (National Renewable Energy Laboratory (NREL), 1998), and as cosmetics and food additives (Sporalore *et al*., 2006).

Fish meal is a primary protein in many formulated diets and also the most expensive component of formulated feeds (Kureshy and Davis, 2000; Davis *et al*., 2008; Martinez-Cordova *et al*., 2010). Fishmeal is used in a variety of feeds including those for poultry, swine, and aquaculture; however the percentage of fishmeal to each of those industries has changed over the past fifty years (Figure 1) (Shepard, 2012; World Bank, 2013). Where poultry in 1960 utilized 48% of the global fishmeal, in 2010, only 5% is used for poultry feed. The vast majority, 73%, now goes to aquaculture feeds (Shepard, 2012).

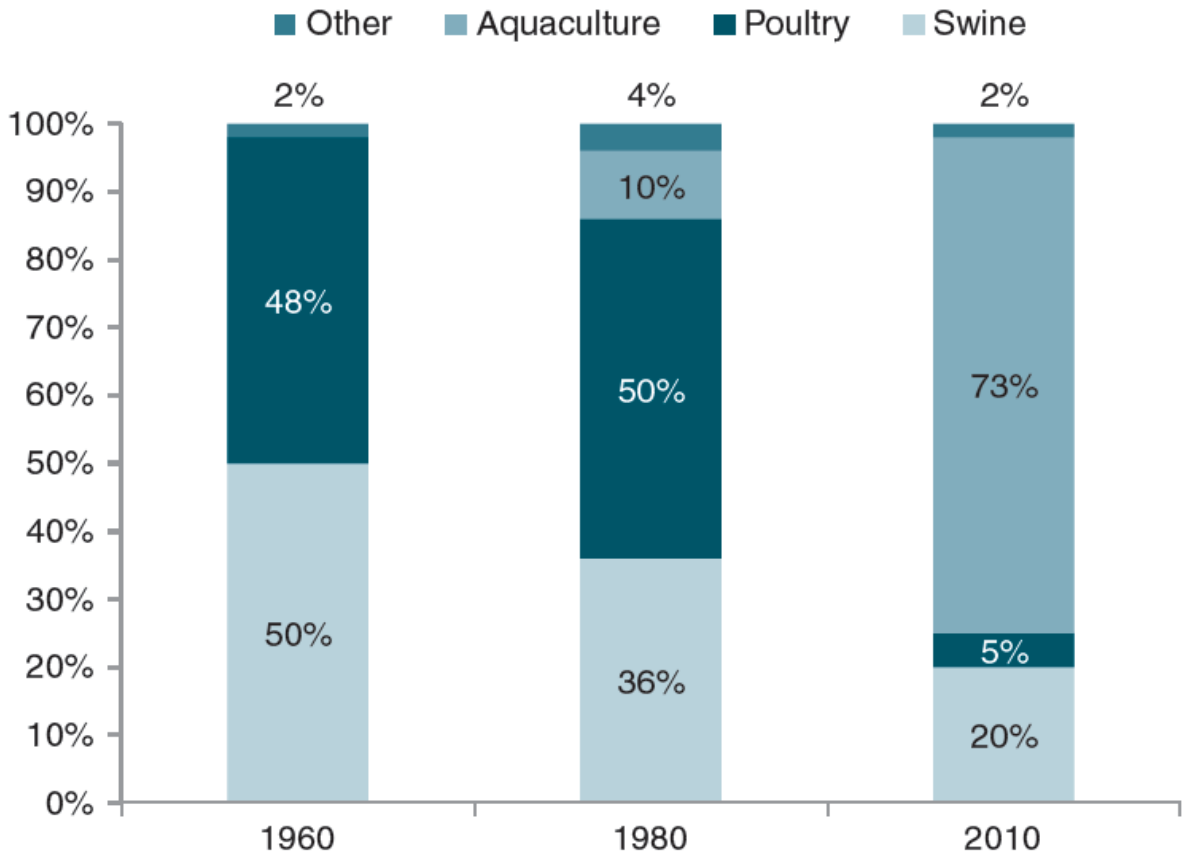

Figure 1. Global fishmeal use 1960-2010 (from World Bank, 2013)

The cost of fishmeal rises and falls yearly (Figure 2), however the cost per ton for fishmeal has increased 58% since 2009 to $1.66K per metric ton (Index Mundi, 2014).

Most shrimp diets are formulated with fish meal and fish oil from pelagic fish fisheries (Samocha *et al.*, 2004; Kureshy and Davis, 2000; Lim *et al.*, 1997). Using biofuel production by-products as a protein and carbohydrate source for Pacific white shrimp, *Litopenaeus vannamei*, has not been extensively investigated although replacing fishmeal with other lower cost protein sources has been considered (Lim *et al.*, 1997; Davis *et al.*, 2004; Samocha *et al.*, 2004; Cruz-Suárez *et al.*, 2007). Finding a way to decrease the cost of fishmeal, may increase the economic competiveness for aquaculturists using such a technology. A reduction or elimination of these ingredients from the shrimp diet would also reduce dependency on offshore fisheries (Cruz-Suárez *et al.*, 2007).

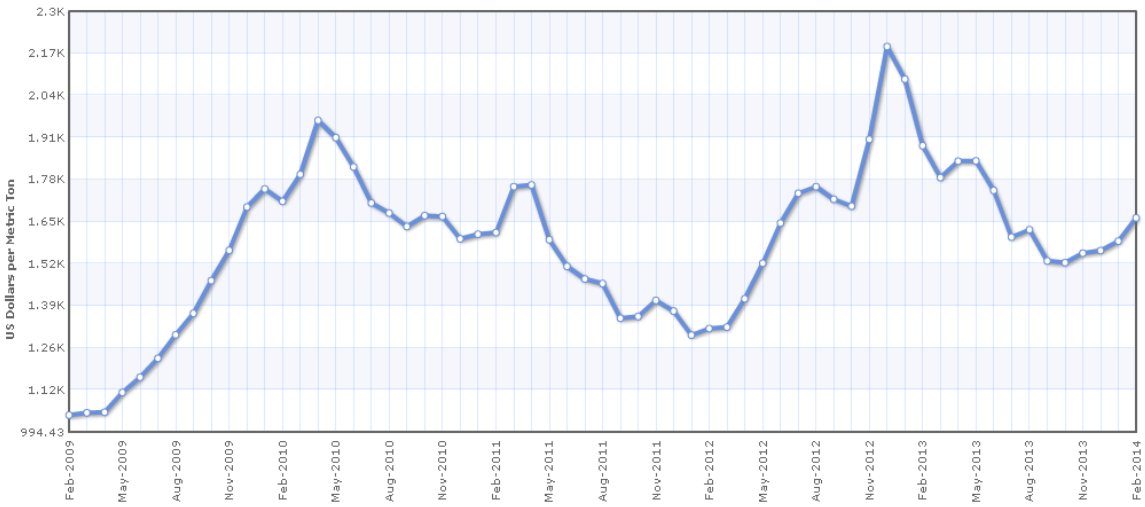

Figure 2. Fishmeal Monthly Price - US Dollars per Metric Ton (from February 2009 to February 2014) Source: Index Mundi, 2014

As the world's population exceeds 6.5 billion, and trends have the population reaching 9 billion by 2050 (U.S. Census, 2012), society puts more pressure on available food resources (Figure 3). It is imperative that research continues to focus on finding sustainable food and energy sources that can meet current and future demands. In 2012, aquaculture provided close to fifty percent of all fish for human

food and this is projected to rise to sixty-two percent by 2030 (Food and Agriculture Organization of the United Nations (FAO), 2014). In 2003, for example, the combined depletion of the world's fisheries reached 132.2 million tons (FAO, 2004). By 2011, that amount increased to 154 million tons (FAO, 2011). Given the projected population growth an additional forty million tons of aquatic food will be required by 2030 to maintain present day levels of per capita consumption (FAO, 2012). By 2050, projections estimate that in order to maintain the current level of per capita consumption, global aquaculture production will need to reach 80 million tons to supplement the global fisheries capture (FAO, 2014).

In concert with the food production crisis brought on by the world's increasing population, an energy crisis may be imminent. Energy consumption is expected to continue to grow rapidly as the world's developing nations continue to require more fuels, primarily those based on fossil hydrocarbons (U.S. Energy Information Administration (EIA), 2013a).

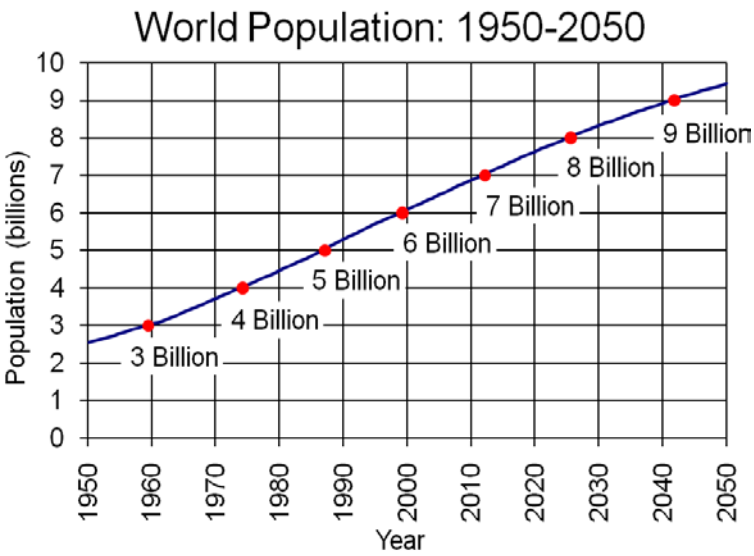

Figure 3. Expected growth of world population: 1950-2050 (from U.S. Census, 2012)

The United States is currently the world's largest producer, consumer and net importer of energy and become a net oil exporter by 2020 (EIA, 2015b). However, oil is a non-renewable resource and the push for renewable sources will continue. The largest increase in sustainable energy will take place in Europe, driven by strong governmental policies including biofuels incentives and mandates that have been implemented by all European Member States (Kampman *et al*., 2013). In 2009, the European Union (EU) set an overall target of renewable energy use of 20% (EIA, 2012). Presently, the share of commercial energy resources in the world by biofuels

is 0.6% and this share is expected to grow to 1.4% by 2030 (EIA, 2012). These two impending crises are presently being addressed separately but it is possible that, there may be a relationship that will provide, at least in part, a synergistic solution to both.

Current research facilitates a shift toward renewable energy sources including the development of bio-diesel from marine algae (LaMonica, 2008; Solecki *et al*. 2013). Harvesting usable oils from marine algae for use in the production of bio-diesel fuel started receiving major funding in the mid 1970's when the United States Department of Energy (US DOE) "Oil-from-Algae" program was started (National Renewable Energy Laboratory (NREL), 1998). Marine algae are primarily composed of carbohydrates, proteins and natural oils. Once the natural oils are harvested from the cells for bio-diesel production, the remaining "by-product" may be viable as a food source for aquaculture species. A major objection or "drawback" of biofuel production has been the disposal of the post-oil-harvest algae (NREL, 1998). Thus as much as fifty (50%) percent of the yield from the algae can be primarily unusable "trash" requiring new sources of disposal (Menetrez, 2012).

Past and current programs have identified marine algae as a rich source of natural oils that can be reacted with simple alcohols (NREL, 1998). The resulting transesterification reaction yields three molecules of biofuel (methyl esters) and a molecule of glycerol for each triacylglyceride (Sheehan *et al*., 1998). Extensive research was conducted by the NREL from 1978 to 1996. The research identified a source of high lipid-content in some species of algae grown in ponds utilizing waste carbon dioxide from coal-fired power plants (NREL, 1998). Most of the algae identified as suitable oil sources were collected from fresh and saltwater in Arizona (NREL, 1998). At the time, the costs of producing algae related to the low income potential of the resulting biofuel were a negative factor for continuing the project, especially as gasoline was inexpensive (EIA, 2014). Since the 1990s, the cost of retail gasoline has risen from $1.07 in 1993 to $3.36 in 2014, but has since dropped to its current (May 2015) price of approximately $2.39 (Figure 4).

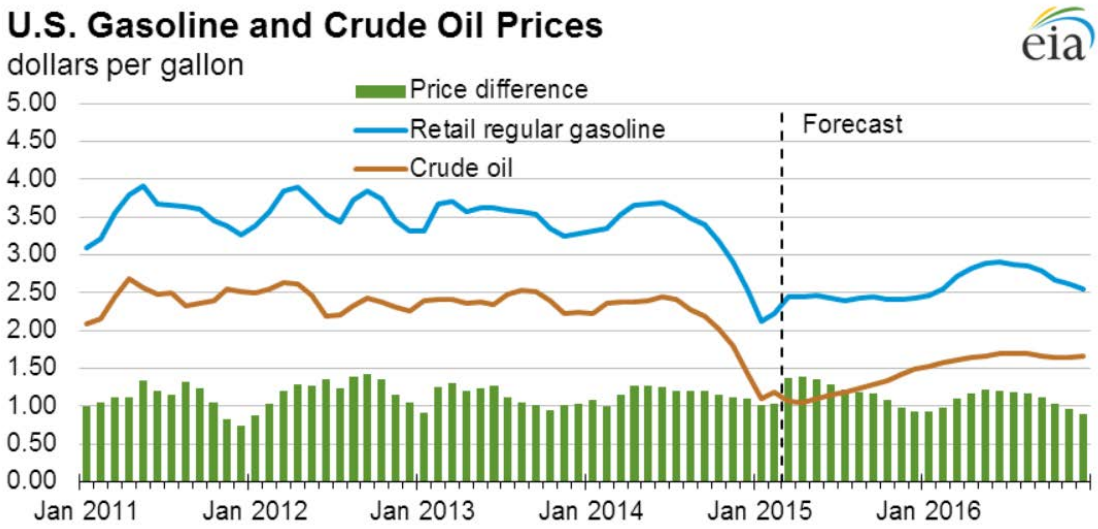

Figure 4. Cost of Gasoline from 1992-2013 (from EIA, 2015a)

Primary protein sources used in aquaculture diets have traditionally come from inexpensive, unsustainable fisheries that historically have kept the price of fish meal inexpensive. Over the past three decades, the cost of fishmeal has increased (Naylor *et al.*, 2000) and research has been carried out to determine suitable replacements (Akiyama, 1988; Lawrence and Castille, 1993; Lim and Dominy, 1990; Samocha *et al*., 2004; Otubusin *et al*., 2009; Rana *et al*., 2009). As early as the 1990s, concern regarding the increasing price of fishmeal was having an effect on the aquaculture market. FAO has expressed concern over the years about the use of marine resources for aquaculture and coined the phrase "fishmeal trap" (Wijkström and New, 1989; New and Wijkström, 1990; New and Wijkström, 2002). The finfish and crustacean aquaculture sector is dependent upon marine capture fisheries for sourcing key dietary inputs such as fish meal and fish oil and has not explored replacement ingredients on a large scale (Tacon and Metian, 2008). Fishmeal was the first ingredient that could lead farmers into a cost-squeeze that could constrain certain forms of aquaculture as it is the most expensive component in feed (Martinez-Cordova *et al*., 2010) and costs per kilogram continue to rise (Index Mundi, 2014). Accordingly, sustainable new protein sources must replace the unsustainable capture proteins that are currently used. FAO has identified a specific need to search for

alternatives for the use of fish protein and highlighted the difficult challenge to move away from fish oils in aquatic animal diets (New and Wijkström, 2002; FAO, 2008).

Herein lies the opportunity to bring a partial solution to two significant population-driven issues, energy and food. The usable natural oils from marine algae address the need for renewable energy source for bio-diesel fuel. With proper treatment and preparation, the remaining portion of the marine algae, the by-product will become a low cost source of needed carbohydrates and protein for aquaculture diets, hence, the possible synergy between bio-diesel production and marine aquaculture food source could potentially serve to alleviate in part both energy and food crises in the making.

1.1 World Seafood Demand

In 2010 the Food and Agriculture Organization of the United Nations (FAO) stated that fish accounted for 15.7 percent of the global population's intake of animal protein and 6.1 percent of all protein consumed (FAO, 2010). In the past five decades, the total and per capita fish food supplies have dramatically increased at an annual rate of 3.1 percent. Additionally, the annual per capita fish consumption grew from an average of 9.9 kg in the 1960s to 11.5 kg in the 1970s, 12.6 kg in the 1980s, 14.4 kg in the 1990s and will likely reach over 17 kg by the late 2000s (FAO, 2012). This high consumption has put fish as the currently most-traded food commodity, worth around $102 billion in 2008 and caused many of the world's fish stocks to become increasingly overexploited and depleted. This "gives cause for concern" (FAO, 2010).

Annual global fish catches, which reached a peak of 86.3 million tons per year in 1996 have since been in a decline (Organization of Economic Co-operation-FAO (OECD-FAO), 2011). By 2008, the annual fish catch dropped to around 79.5 million tons (OECD-FAO, 2011). The downward trend has been explained by some scientists as a result from over-fishing and a replenishing of fish stocks, others believe the data prove fish stocks are already overexploited or depleted. If capture fisheries

remain stagnant, the harvest shortfall will need to be made by up aquaculture (Figure 5). In 2011, the OECD-FAO which is a 34-member organization to provide information to help governments foster prosperity and fight poverty through

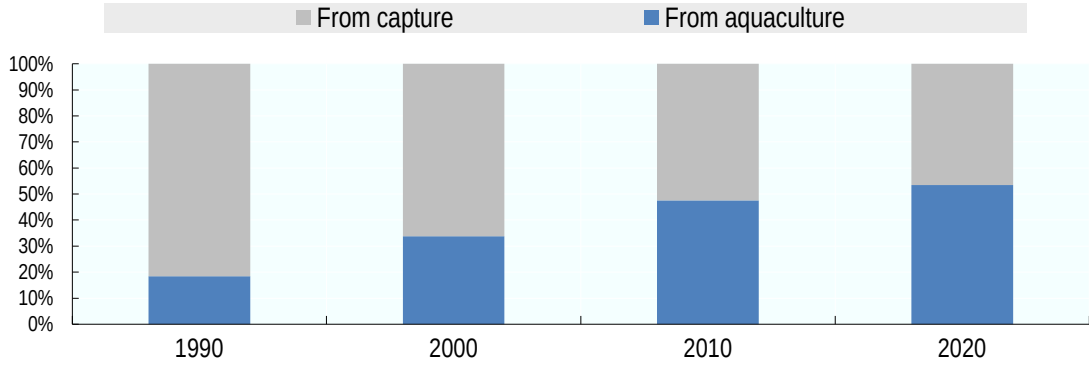

Figure 5. Increasing role of aquaculture in fish consumption (from OECD-FAO, 2011)

economic growth and financial stability, and the FAO of the United Nations forecasted that the average world price for captured species to increase by 23% and aquaculture species by 50% by 2020 compared to 2008-2009 prices (OECD-FAO, 2011).

1.2 World Energy Consumption

Global energy consumption is projected to rise by 35 to 50 percent between years 2009 and 2035 (EIA, 2014; ExxonMobil, 2015). Most of the growth occurs in emerging economies outside the OECD, especially in non-OECD Asia. Total non-OECD energy use increases by 84 percent, compared with a 14-percent increase in the developed OECD nations (OECD-FAO, 2011).

As the use of sources of energy continues to increase, market forces are expected that oil prices will remain relatively high in the long term. The United States Energy Information Administration (EIA) collects and disseminates information about energy information and policy. In 2014, EIA projected that a barrel of oil could increase to as high as $150 by 2025 (EIA, 2014). High energy prices and concerns about the environmental consequences of greenhouse gas emissions led a number of national

governments to provide incentives to support the development of alternative energy sources, making renewable energy the world's fastest-growing source of energy (OECD-FAO, 2011). The International Energy Agency, which is an organization that provides energy policy guidance, has projected that power generation from hydro, wind, solar, and other renewable sources worldwide will continue to grow to the point that they will surpass energy generation from nuclear plants in the foreseeable future (IEA, 2013).

1.3 United States of America Energy Consumption

Increasing population affects energy use through increases in housing, transportation, economic activity, and workplace activity. The U.S. population is projected to increase by 0.9% per year from 2011 to 2040 (EIA, 2013a). As previously mentioned, global consumption is projected to increase by 35 percent or more by 2040; however, during that same period, energy consumption is forecasted to decline in the U.S. by 5% from 2010 to 2040 even as gross domestic product doubles and population rises to approximately 375 million people (ExxonMobil, 2015). The decline in energy use per capita is forecast to be due to using energy more efficiently in homes, businesses, transportation, and in the generation of electricity. From the 1970s through 2008, typical energy use per person was at 320 million Btu per person. It is estimated that energy use in 2034 will drop to 270 million Btus per person in the US, which was the level in 1963 (EIA, 2013a). However, even though this is the trend in developed nations, other nations such as China and Brazil, are developing plans to provide electricity to their entire populations using traditional fossil fuels for energy (EIA, 2014).

1.4 Renewable Energy

The EIA (2014) includes seven fuel types as renewable energy sources:

- Hydroelectric
- Geothermal
- Solar

- Wind
- Wood biomass (includes wood and wood wastes)
- Ethanol
- Biodiesel

EIA (2014) projects that total renewables used for electricity and heat generation will grow by 2.2% in 2014. Conventional hydropower generation is projected to fall by 4.2%, while non-hydropower renewables rise by 5.6%. Non-hydropower renewables generation surpassed hydropower on an annual basis for the first time in 2014. In 2015, total renewables consumption for electric power and heat generation increased by 4.6%, as a result of a 4.3% increase in hydropower and a 4.7% increase in non-hydropower renewables (EIA, 2014).

In the U.S., non-hydroelectric renewable generating capacity, supported in part by Federal tax credits, has grown at a faster rate than fossil fuel capacity (EIA, 2011). It is estimated that total non-hydroelectric renewable capacity will increase from 47 gigawatts in 2009 to 100 gigawatts in 2035 (EIA, 2011). The largest increase is in wind-powered generating capacity; however, as the Federal Production Tax Credit, expired at the end of 2013, the trend may not continue at the same pace as previous years (Patel, 2014).

As a result of the Energy Independence and Security Act of 2007 which established the Renewable Fuel Standard, biofuels production is expected to increase by almost 1.5 million barrel per day, with ethanol accounting for the largest share of the increase (EIA, 2013b). Ethanol production is expected to increase by more than 800,000 barrels per day from 2009 to 2035, displacing approximately 12 percent of gasoline demand in 2035 on an energy-equivalent basis (EIA, 2011).

1.5 Biofuel Production and Sales

As human population increases, it is critical that additional sources of energy be found. Currently, nearly all renewable energy sources (e.g. hydroelectric, solar, wind, tidal, geothermal) are developed to provide energy to the electricity market; however,

fuels fill a much larger share of the global energy demand (~66%) (Scheck *et al*., 2008). Biodiesel is currently produced from oil synthesized by conventional fuel crops (first generation biofuels) or microalgae (second generation biofuels) that harvest the sun's energy and store it as chemical energy (NREL, 1998; Scheck *et al*., 2008). It is estimated that the monthly US biofuel production rose to 113 million gallons in June 2013, up from 111 million gallons in May (EIA, 2013b). Seventy percent of that production came from the Midwest region. EIA (2013b) reported that there are 110 biodiesel plants online with a capacity of 175 million gallons per month; thus; collectively they are operating at 64% percent of capacity based on June 2013 production.

In June 2013, producer sales included 77 million gallons sold as B100 (100% biodiesel) and an additional 36 million gallons of B100 blends with diesel fuel derived from petroleum. The biodiesel was derived primarily from soybean oil (461 million pounds) followed by corn oil (98 million pounds), yellow grease (i.e., used cooking oil; 93 million pounds), and tallow (i.e., rendered fat; 54 million pounds) (EIA, 2013b). Accounting for all sources, a total of 873 million pounds of feedstocks were utilized to produce biodiesel in June 2013 (EIA, 2013b).

1.6 Biofuel Production from Algae

The production of biofuel from algae can be divided into three primary steps and the resulting product can be further refined to yield ethanol, methane, hydrogen, biodiesel and oil (Miao and Wu, 2006; Sayadi *et al*., 2011; Collet *et al*., 2014). As illustrated in Figure 6, the process begins as the inputs are determined. For most species of algae, growth is highly dependent on the availability of suitable nutrients and light, for most cases. Algae have been suggested as a candidate for biofuel production due to their higher photosynthetic efficiency, higher biomass production, reduced footprint, and faster growth as compared to terrestrial crops (Miao and Wu, 2006). Following the extraction of the high value oils, the remaining algal biomass could be

harvested, dried, and co-fired with coal powered electrical power plants (Kadam, 2002).

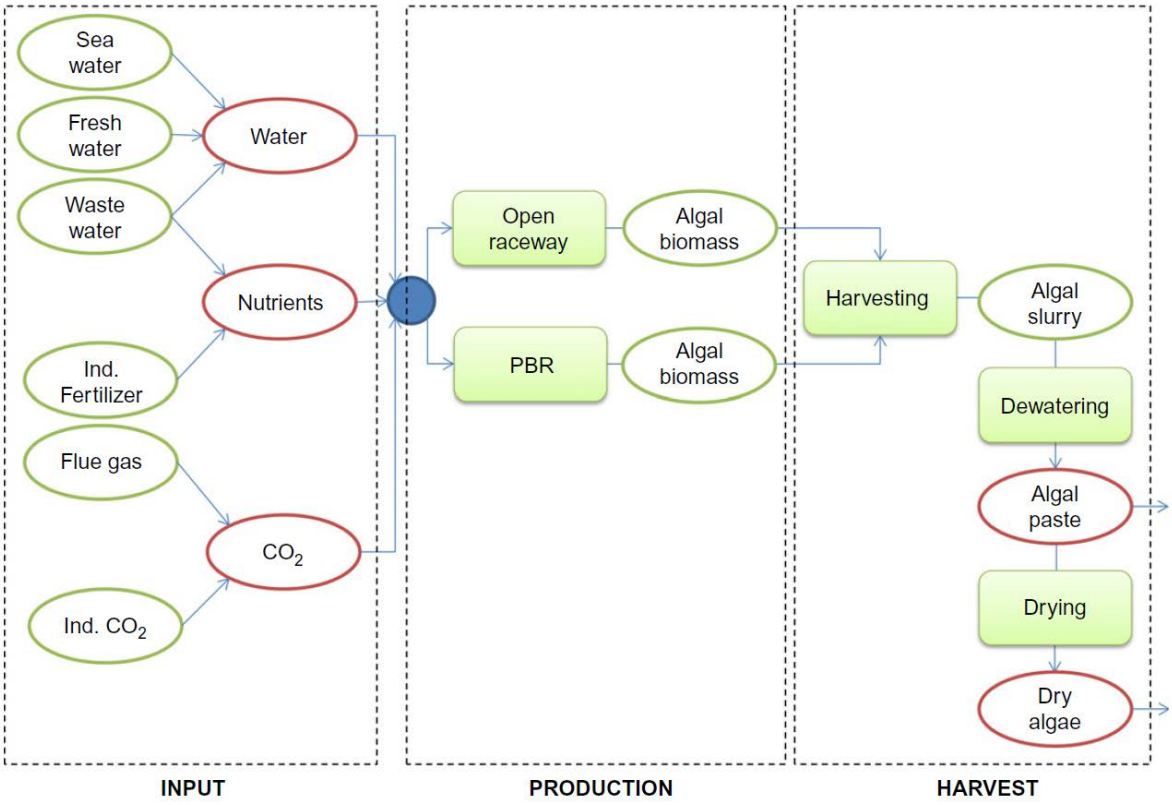

Figure 6. Input, production, and harvest flowchart for biofuel production from algae (from Collet *et al.*, 2014)

1.6.1 Production of Algae

Algae for food and for high value products such as astaxanthin, phycibiliprotien, Beta-carotene, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) has already been done profitably and the market continues to grow (Milledge, 2012). However, the economic margin of producing algae for biofuels is much smaller as the biofuel market value is much lower (Scheck *et al.*, 2008). There are several different methods of cultivating algae and each has advantages and disadvantages (See 1.6.3 Algae Culture Systems). In addition to optimizing the species of algae, it is important to use a production method that can produce large quantities of algae and high levels

of lipids. The optimization of culture conditions is an area of research that is critical to insure the growth of the industry (NREL, 1998; Schenk *et al*., 2008; Lundquist *et al*., 2010).

1.6.2 Lipid Production by Microalgae in Nature

Under normal growth conditions, most algal species have a lipid content of approximately 10-30% dry weight (Sheehan *et al*., 1998; Schenk *et al*., 2008; Lundquist *et al*., 2010). However, during certain stress events (e.g., culturing cells in a nutrient limited environment) the cells cease dividing and algae produce higher amounts of lipids (Table 1) (Sheehan *et al*., 1998; Lundquist *et al*., 2010; Collet *et al*, 2014).

Table 1. Percentage of dry weight lipids in algae cultured in nutrient limited environments (after Sheen *et al*., 1998)

Algal Species	Percentage of Dry Weight Lipids
Nannochloropsis sp.	31-68
Botrytococcus braunii	25-75
Schizochytrium sp.	50-77
Neochloris oleaabundans	35-54
Nitschia sp.	45-47
Chlorella vulgaris	24-65

With the exception of *Nannochloropsis* mentioned in the table above, most cultured algae do not produce and store large amounts of triglycerides while actively growing and must be stressed in order to initiate higher oil production (Lundquist *et al*., 2010).

1.6.3 Algae Culture Systems

Most culture systems are an open system, a closed bioreactor, or a hybrid of the two (Weissman *et al*., 1988; Schenk *et al*., 2008; Collet *et al*, 2014). The traditional method of cultivating algae for bulk use is with open pond systems. These systems can be built and operated economically and offer many advantages (Weissman *et al*.,

1988; Collet *et al*, 2014). The difficulty in using this system usually comes from the difficulty in controlling the algae in the ponds. Having a monoculture of a high lipid yielding algae is virtually impossible; however, that is not necessarily critical as the system benefits from high production of algae for less cost than closed bioreactor systems. A system that has been designed to maximize algae production incorporates an oval pond with a paddlewheel (Figure 6). The paddlewheel is used to create a constant flow mixing the layers of the pond throughout the growout cycle ensuring that algal cells come in contact with sunrays on a periodic basis (Scheck *et al*., 2008). Even though these systems are shallow (15-20 cm), biomass concentrations of 1 g dry weight per liter and productivities of 60-100 mg L^{-1} day^{-1} (i.e. 10-25 g m^{-2} day^{-1}) are possible. As many culture systems are outdoor and exposed to the normal fluctuations of temperature and light, keeping up productivity is a challenge throughout the year (Scheck *et al*., 2008).

Figure 7. Example of oval production tanks at Israel Electric Company in Ashkelon, Israel (from National Renewable Energy Laboratory; www.NREL.gov)

Closed bioreactor systems save water, energy and chemicals, and provides the technicians greater control over the algal species as contamination can be kept to a minimum. Closed systems can produce more dry weight of algae than the traditional pond systems (Carlozzi, 2003). Closed bioreactor systems can be divided into four main categories: plate; tubular; annular; and plate airlift (Figure 8; Schenk et *al*., 2008). These systems are excellent in maintaining axenic cultures however the cost

of initial construction and maintenance may limit their value as compared to open water systems like the ones previously mentioned (Schenk et *al*., 2008).

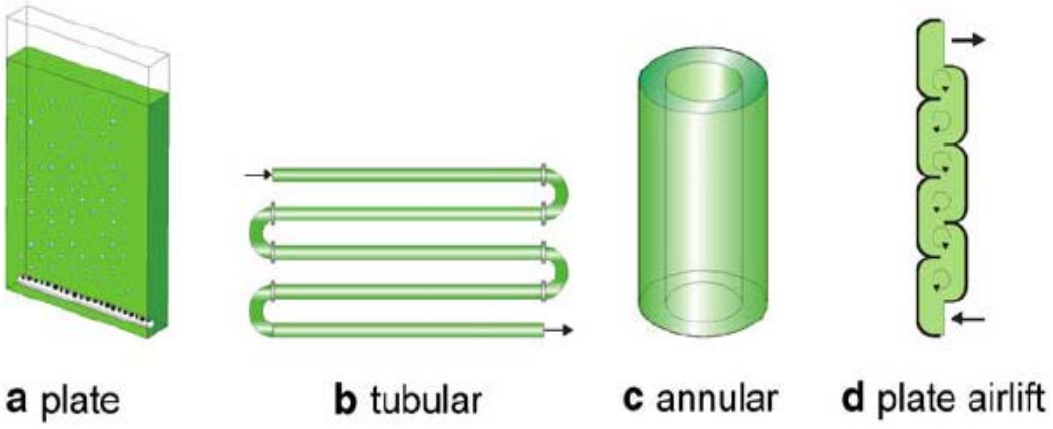

Figure 8. Closed bioreactor systems (from Schenk *et al*., 2008)

Typical systems using tubular reactors in a fence-like construction can produce up to 47 g dry weight m^{-2} day^{-1} (Carlozzi, 2003). More advanced systems, such as the "3DMS-Reactor" at the Massachusetts Institute of Technology exhibits an average of 98 g dry weight m^{-2} day^{-1} (Pulz, 2001; 2007).

1.6.4 Harvesting and Extraction Methods

The harvesting processes and extraction methods are viewed as major limiting factors on the growth of this industry as present processes are energy dependent (Molina Grima *et al*., 2003). The harvesting and extraction methods can represent 20-30% of total production costs (Molina Grima *et al*., 2003).

Lipids can be extracted from algal cells in several ways. The first step in the extraction process is to reduce the water content and concentrate the algae cells. The most common harvesting methods are micro-screening or filtration, sedimentation, centrifugation, and flocculation (Uduman *et al*., 2010). Recent research shows promise using suspended air flotation for algal harvesting (Wiley *et al*., 2009). Choosing the appropriate method or combination of methods can be influenced by the species chosen for culture. Certain species are easier to harvest than others as their

density, size, and shape impact the success of harvesting (Benemann and Oswald, 1996). For example, the cyanobacterium *Spirulina*'s long spiral shape naturally lends itself to micro-screen harvesting method (Scheck *et al*., 2008). Filtration is a method that can be applied at the laboratory scale but suffers drawbacks when applied to large-scale operations due to membrane-clogging, the formation of compressible filter cakes, and high maintenance costs (Scheck *et al*., 2008).

Sedimentation can be used but it is time-consuming and requires a large amount of space to produce commercially viable quantities of algae. The cost of centrifugation is expensive and at this time it may only be a commercially viable solution to reduce slurry (10-20 g/l) to an algal paste (100-200 g/l) and not for the entire extraction method (Scheck *et al*., 2008).

Flocculation to concentrate the algal cells is commonly used in wastewater operations where an inorganic chemical such as alum, ferric oxide, and lime are used. These chemicals can be cost prohibitive. Organic cationic polyelectrolyte flocculants are preferred as much less is needed (Molina Grima *et al*., 2006). Natural bioflocculation or spontaneous flocculation is the most promising economically and has been seen to be effective for some species. Some species naturally flocculate and others in response to certain environmental conditions, such as nitrogen stress, pH, and level of dissolved oxygen, flocculate which makes the harvesting process easier (Benemann and Oswald, 1996).

Once the cells are concentrated, the next step is to extract the oil. The extraction can be done using various methods including bead mill homogenizers, freezing, alkali and organic solvents, osmotic shocks, bead milling (Molina Grima *et al*., 2003) and mechanical expeller press (Topare *et al*., 2011). Each of these methods has advantages and disadvantages which include, for example, the efficiency of oil removal and the cost of operation. Topare *et al.* found that expeller presses could recover 75% of the oil from algae during their trials (2011). This method is less expensive than utilizing the solvent extraction method but as solvent extraction

method recovers almost all of the oils (99.5%), the cost savings of using expeller presses would need to be analyzed closely (Topare *et al*., 2011).

1.6.5 Economic Feasibility of Microalgal Biodiesel

With current technology, the potential of biofuels production is cost competitive when crude oil prices are between $40 to $60 per barrel (Tredici, 2003; Schenk *et al*., 2008). Recent studies estimate that the microalgae oil-based technologies have similar environmental effects as compared to other vegetable oils but the profitability still needs to be refined (Torres *et al*., 2013). Numerous life cycle assessments have been carried out and each identified that the large number of variables including size of facility, harvesting method, and oil extraction technique can impact the break-even price making forecasting very difficult (Passell *et al.*, 2013; Torres *et al*., 2013). First generation biofuels focused on conventional crops such as soybean, jatropha, and oil palm. As these crops often required intensive fertilizer use, machinery for cultivation and refining, and transportation, they are not considered carbon-neutral crops. Second generation biofuels, of which microalgae is part of, are more water-efficient and require much less arable land (Table 2) than conventional crops. Microalgae are already reported to produce 15-300 times more oil for biodiesel than traditional crops used in first generation biofuels (Christi, 2007). Certain industry groups believe that algae biodiesel could be competitive with oil in seven years while others believe it may come closer to three years (Feldman, 2010).

Table 2. Comparison of crop-dependent biodiesel production efficiencies from plant oils (after Scheck *et al.*, 2008)

Plant Source	Biodiesel (L/ha/year)	Area to produce global oil demand (hectares x 10^6)	Area required as percent of global land mass	Area as percent of global arable land
Cotton	325	15,002	100.7	756.9
Soybean	446	10,932	71.4	551.6
Mustard seed	572	8,524	57.2	430.1
Sunflower	952	5,121	34.4	258.4
Rapeseed/canola	1,190	4,097	27.5	206.7
Jatropha	1,892	2,577	17.3	130
Oil Palm	5,950	819	5.5	41.3
Algae w/30% TAG[1]	12,000	406	2.7	20.5
Algae w/50% TAG[2]	98,500	49	0.3	2.5

[1] Algae with 10 g m^{-2} day^{-1} at 30% triacylglycerol (TAG)

[2] Algae with 50 g m^{-2} day^{-1} at 50% triacylglycerol

1.7 Aquaculture

Aquaculture started when the first fish was caught and placed in a ditch or pond, fed, and then harvested after a period of time. Although it is difficult to determine exactly where aquaculture began, historical records lead us to locations throughout the world. An Egyptian bas-relief on the tomb of Aktihetep (2500 B.C.) depicts what appear to be men capturing fish, possibly tilapia, from a pond. In China, carp were grown around 500 B.C. The "first fish farmer" Wen Fang, founder of the Chou dynasty, built ponds and kept records on the growth and behavior of fish (Landau, 1992).

The culture of shrimp is attributed to Motosaku Fujinaga as he first successfully spawned and partially reared marine penaeid shrimp in 1934 (Stickney and Treece, 2012). His techniques were adopted in the United States in the 1950s and 1960s. His visit to the National Marine Fisheries Laboratory in Galveston in 1963 helped promote future research (Nash, 2010). J.B. Panaouse discovered one of the key advances that helped successfully spawn penaied in the 1940s when he discovered

that maturation could be induced with the removal of the eyestalks which is critical to crustacean endocrine activity (Landau, 1992).

Aquaculture has developed into the world's fastest-growing source of animal protein and currently provides nearly half of all fish consumed globally (FAO, 2010). The pace of growth is high as global production of fish from aquaculture grew more than 60 percent between 2000 and 2008, from 32.4 million tons to 52.5 million tons (FAO, 2010).

1.7.1 Shrimp Aquaculture and the Environment

During the 1990s, shrimp farming was the fastest growing segment of aquaculture in the US; however, its growth was marred by being associated with negative environmental impacts (Boyd and Clay, 2002). These environmental impacts have forced US farmers to meet acceptable pollutant levels in discharge which in some ways has slowed expansion of the industry (Lawrence *et al*., 2001). Efforts to move shrimp farming away from coastlines has been relatively successful with farms advancing the science needed to raise shrimp in lower salinity as the cost to have seawater inland is cost prohibitive in most cases (Davis *et al.*, 2002)

Growout operations begin at the time at which larvae are stocked into open ponds (lined or unlined) or runways (outside or inside raceways). These operations are classified by stocking densities which is normally described by the number of seed stock per hectare or number of seed stock per cubic meter (Briggs *et al*., 2004). There are four classifications: extensive, semi-intensive, intensive and super-intensive (SMEDA, 2007; Bojórquez-Mascareño and Soto-Jiménez, 2013). Table 3 provides the division for each of the classifications. The classification has direct implications on the feed regime that is needed to ensure high survivability and promote growth. As an operation is more intensive, the natural foods (phytoplankton, zooplankton), bio-floc, and detritus that shrimp would typically feed on for survival will be inadequate without supplement of formulated feed. Additionally, the need for water

exchange or water treatment, as well as possible aeration, increases as the stocking density increases (SMEDA, 2007).

Table 3. Stocking densities of growout operations (Briggs *et al.*, 2004; Carvajal-Valdes *et al.*, 2012)

Classification	Seed Stock per Hectare (x 1000)	Yields per Hectare per Year (kilograms)
Extensive	<15	50-500
Semi-Intensive	15-35	500-5,000
Intensive	100-150	5,000-20,000
Super-Intensive	>150	20,000-100,000

Feeds can contribute a significant amount of enriching nutrients in effluent that could necessitate the formulation of "environmentally friendly" or "least polluting" feeds to help meet environmental standards. Velasco *et al*. (1998) demonstrated the correlation between dietary protein and the accumulation of inorganic nitrogen in culture water. They also observed that diets that maximize protein utilization for growth as opposed to energy needs lead to the reduction of nitrogenous compounds in aquaculture effluent. Protein levels in feed also must be optimized to reduce production costs as protein accounts for the majority of feed content and expense (Cordova-Murueta and Garcia-Carreno, 2002) and feed costs currently account for the majority of production costs (Akiyama *et al*., 1992; Otubusin *et al*., 2009; Rana *et al.*, 2009). Feed has been the single largest operating cost in intensive aquaculture (Otubusin *et al*., 2009). Shrimp farmers also have begun to increase stocking densities in ponds and raceways to intensive or even super intensive levels to deal with the reduction in shrimp prices (Cuzon *et al.*, 2003). Such intensification places the nutritional burden on supplemented feed as opposed to natural productivity and forces nutritionists to formulate feeds to contain the proper balance of energy, protein, minerals and vitamins while preserving the cost efficiencies realized through intensification. Feed formulators in turn look

to researchers to provide them with optimal nutrient levels to meet these challenges (Lim, 1997; Kureshy and Davis, 2000; Samocha *et al*., 2003; Davis *et al*., 2004; Cruz-Suárez *et al.*, 2007; Otubusin *et al*., 2009). Dietary protein requirements have been estimated by feeding trials in which graded levels of protein are fed to apparent satiation or in excess, to determine growth response (typically, weight gain) under controlled or observed environmental conditions (Kureshy and Davis, 2000). Protein requirements of an animal can be defined as the minimum or the maximum of protein needed per animal per day (Guillaume, 1997). The term Energy-to-Protein (E:P) is often used to quantify dietary requirements. Studies have suggested protein requirements of juvenile *L. vannamei* range from an as-fed dietary inclusion level of 15%, with an energy to protein (E:P) ratio of 119.58 kJ/g protein (Aranyakananda, 1995), to approximately 30% of diet, with a dietary E:P ratio of 41.86 kJ/g protein (Cousin *et al*., 1991), to greater than 36% of diet (Smith *et al*., 1985) and even greater than 40% of diet (Colvin and Brand, 1977). These variations are not surprising considering that protein requirements can vary with age, size, physiological status, growth rate and dietary characteristics such as E:P ratio (Colvin and Brand, 1977; Guillaume, 1997; Pedrazzoli *et al*., 1998). Differences also may arise as these studies utilized an ad-libitum feeding method which could allow shrimp to increase their feed intake to negate the effect of a low protein diet and lead to substantial variation in dietary E:P requirement (Kureshy and Davis, 2002).

Table 4. Scientific Classification

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class:	Malacostraca
Subclass	Eumalacostraca
Superorder	Eucarida
Order:	Decapoda
Suborder:	Dendrobranchiata
Superfamily:	Penaeoidea
Family:	Penaeidae

1.8 Family Penaeidae

Penaeidae is a family of marine crustacean in the suborder Dendrobranchiata and superfamily Penaeoidea, and are often referred to as *penaeid shrimp* or *penaeid prawn* (Table 4). The suborder Dendrobranchiata contains over 500 species of

shrimp that are found from shallow water of the tropics to a depth of over 1000 m (Pérez-Farfante and Kingsley, 1997). The history of attempts to classify this group is quite long and often taxonomists and geneticists disagreed and some dissent remains (Tavares and Martin, 2009). Nonetheless, within the family Pennaeidae Pérez-Farfante and Kensley (1997) subdivided the genus *Penaeus* into *Farfantepenaeus*, *Fenneropenaeus*, *Litopenaeus*, *Marsupenaeus*, *Mesopenaeus*, *Metopenaeus*, and *Penaeus* and this is commonly accepted.

1.8.1 Taxonomic Classification

Previous authors (Burkenroad, 1981; Pérez-Farfante and Kensley, 1997; Dixon *et al*., 2003) defined the suborder by the following characteristics:

(1) the presence of dendrobranchiate gills;
(2) the appearance during development of pleurobranchiae after the arthrobranchiae and podobranchiae;
(3) the possession of (usually) chelae on the first three pairs of pereiopods;
(4) the second pleomere with pleura that do not overlap those of the first;
(5) prominent hinges between the pleomeres;
(6) eggs that are released directly into the water (as opposed to being carried by females) and that hatch as a lecithotrophic nauplius or protozoea;
(7) the presence of a petasma in males; and
(8) pleopods that lack an appendix interna, with the exception of vestigial structures found in some males.

The order decapods (ten legs, or pair of legs) are united by having a carapace enclosing the brachial chambers. The first pairs of thoracopods have been modified as maxillipeds to assist in feeding (Dixon *et al*., 2003).

1.8.2 Principal Cultured Species

As per the Food and Agricultural Organization of the United Nations, there are 342 actual or potentially significant commercial species of shrimp (FAO, 2011). This

number includes shrimp used directly for human consumption, species used for feeding other cultured aquaculture species, and those which are considered to have some commercial value (FAO, 2012). Of the 109 species of the family penaeidae, only six are cultured worldwide in quantity (Table 5):

1. Pacific Whiteleg Shrimp (*Litopenaeus vannamei*)
2. Giant Tiger Shrimp (*Penaeus mondon*)
3. Brown Shrimp (*Farfantepenaeus duorarum*)
4. Western Blue Shrimp (*Penaeus stylirostris*)
5. Japanese Kuruma Shrimp (*Penaeus japonicus*)
6. Indian White Shrimp (*Penaeus indicus*)

In the United States, there have been thirteen different principal species cultured, however, most of the market is dominated by *L. vannamei* and *L. setiferus* (Table 5; Treece and Fox, 1993).

Table 5. Principal species cultured in the United States (after Treece and Fox, 1993)

West Coast	East Coast	Exotic
L. vannamei	*L. setiferus*	*P. monodon*
L. stylirostris	*F. duorarum*	*P. indicus*
F. brevirostris	*F. aztecus*	*P. japonicus*
L. occidentalis	*L. schmitti*	*P. semisulcatus*
	F. brasiliensis	

1.8.3 Litopenaeus vannamei (Boone 1931)

The Pacific whiteleg shrimp, *L. vannamei*, formerly *Penaeus vannamei*, is endemic to the eastern Pacific Ocean. Also known as the Pacific white shrimp, its range stretches from Sonora in Mexico to northern Peru (Table 6). It is the most popular farmed species in the world with annual world production in 2010 of over 2.7 million tons (Alcivar-Warren *et al.*, 2007; FAO, 2012). Latin America, Brazil, India, China, Thailand, Indonesia, United States of America, and several countries in Africa have focused their farms on *L. vannamei* production. *Litopenaeus vannamei* grows to a

maximum length of 230 millimeters, with a carapace length of 90 mm. Adults live in the ocean, at depths of up to 72 meters, while juveniles live in estuaries. It is restricted to areas where the water temperature remains above 20°C (68°F) throughout the year. Production of *L. vannamei* is limited by its susceptibility to various diseases (see section 1.7.1) (FAO, 2014). Good hatchery, maturation, and growout protocols have been established (Hopkins *et al*., 1994; McIntosh *et al*., 2000; 2001) allowing *L. vannamei* to be a primary species for culture.

Table 6. Summary of Common Species

Species	Common Name	Distribution	Size (mm)	Thelycum	Rostrum
Litopenaeus vannamei (Boone 1931)	Whiteleg shrimp; Pacific White Shrimp	Eastern Pacific	230 (♂)(♀)	Open	7-10 teeth on dorsal; 2-4 teeth on ventral
Penaeus monodon (Fabricus 1798)	Tiger Shrimp	Indo-west Pacific, Sea of Japan	330 (♂)(♀)	Closed	7-8 teeth on dorsal; 3-4 teeth on ventral
Farfantepenaeus duorarum (Burkenroad 1939)	Brown Shrimp	Western Atlantic from Mid-Atlantic U.S. to Cuidad Campeche, Mexico	269 (♂) 288 (♀)	Closed	6-7 teeth on dorsal 1-3 teeth on ventral
Penaeus indicus (H. Milne Edwards 1837)	Indian White Prawn	East Africa, South Africa, India, Bangladesh, Indo-West Pacific, Southern China and the Northern coast of Australia	184 (♂) 230 (♀)	Closed	7-9 teeth on dorsal; 3-6 teeth on ventral
Penaeus japonicus (Bate 1888)	Kuruma Shrimp	Indo-West Pacific, the east and southeast Africa, and the Red Sea	190 (♂) 225 (♀)	Closed	7-11 teeth on dorsal; 1 tooth on ventral
Litopenaeus stylirostris (Stimpson 1874)	White Shrimp; Southern Shrimp	Western Atlantic from Mid-Atlantic U.S. to Cuidad Campeche, Mexico	197 (♂)(♀)	Open	5-11 teeth on dorsal; 2 teeth on ventral

1.9 Challenges to shrimp production

In addition to the basic premise that shrimp production should be carried out in an environmentally friendly manner and comply with international standards to meet food safety requirements, there are two primary challenges to shrimp production: (1) the cost of production in intensive farming is hampered by the high cost of feed; and

(2) in all types of farming, shrimp aquaculture has been challenged by the incidence of disease and its impact on the final crop (Cuzon *et al*., 2004). In Section 1.9.1, five penaied diseases are summarized (Lightner, 1999; 2003; Pantoja *et al*., 2008).

1.9.1 Penaied Diseases

Even with all of the advances that were made in the culturing, nutrition, and marketing of the species, disease outbreaks during the late 1980s and 1990s caused great concern that the industry would collapse (Flegel *et al.*, 2008). Until the late 1990s, most post-larvae were reared after the capture of wild broodstock (Krantz, 1976). Research that was conducted at the University of Hawaii – Oceanic Institute, produced commercially available and genetically superior post-larvae for the industry (Argue *et al*., 2002; Moss *et al*., 2005; Flegel, 2009). The production of specific-pathogen-free (SPF) broodstock and post-larvae become the standard for pond stockings throughout the world. Companies that did not, and even some that did, stock with SPF or specific-pathogen-resistant (SPR) shrimp were decimated by several diseases (Flegel *et al.*, 2008). White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Taura Syndrome (TS), Infectious Hypodermal & Haematopoietic necrosis (IHHNV), Baculoviral Midgut Gland Necrosis (BMN) and vibriosis have all generated a considerable amount of attention in regards to best management practices which include starting with SPF post-larvae, minimal-to-zero pond water exchange, and feed management (Lightner, 1999; 2003; Argue *et al*., 2002). Table 7 provides descriptions for the five diseases as described in the European Community Reference Laboratory for Crustacean Diseases (2013).

Table 7. Summary of common penaeid diseases (Lightner, 1996; Pantoja *et al*., 2008)

Disease	Description	Stability	Geographical Distribution	Mortality
White Spot Syndrome Virus (WSSV)	Rod-shaped to elliptical; measure 80-120 mm x 250-380 mm	Viable for at least 30 days at 30°C (laboratory) and 3-4 days at 30°C (pond)	East, South-East, and South Asia; North, South, and Central America	High; outbreaks may be induced by stressors (salinity change, low water temperature)
Yellow Head Virus (YHV)	Rod-shaped with helical nulceus	Viable in aerated seawater for up to 72 hours	East, South-East, and South Asia; North, South, and Central America	100% mortality in ponds within 3 days of the first appearance of clinical signs
Taura Syndrome (TS)	Non-enveloped icosahedrons virus particles measuring 30-32 nm	Up to 48 hours in the feces passed by wild or captive sea gulls after consuming TS infected shrimp carcasses	East, South-East, and South Asia; North, South, and Central America	40-90% mortality
Infectious Hypodermal & Haematopoietic Necrosis Virus (IHHNV)	Small (22 nm average diameter), single strand DNA-containing parvovirus	IHHN virus in infected shrimp tissues remains infectious after five years of storage at –20°C and after 10 years at –80°C	East, South-East, and South Asia; North, South, and Central America; Israel; Australia	80-90% cumulative mortalities in postlarvae and juveniles
Vibrosis	Curved, rod shaped with polar flagella with sheaths	High stability	Ubiquitous	Insignificant to 100%

1.9.2 Costs of Production

Overall market acceptance and a well understood life-cycle has continued to promote further capital investments into *L. vannamei* farming operations (Bray *et al*., 1994; Boyd, 2001; Hedlund, 2007; Alday-Sanz, 2010). Consequently, with the expansion of the farming industry, the price has decreased, which has made it difficult for smaller famers to survive in the market. FAO releases information yearly on the cost of production but determining accurate production costs is difficult and varies depending on many factors (FAO, 2014). Post larval (PL) shrimp production varies from country to country due to operational costs and these costs are highly variable (Davis *et al*., 2008). Post larval shrimp follow a nomenclature to define the number of days since the animal has completed their final metamorphosis. For example, PL_{10}

would be used to designate an animal that completed their final metamorphosis ten days prior. In the US, the average cost per 1000 PL is $0.5–1.0. The average price in China can be as low as $0.4/1,000 for $PL_{8–10}$ and up to $1.5–3.0/1,000 for PL_{12} in other parts of Asia. *Vannamei* are often preferred for farming due to lower feed costs and higher stocking levels which result in mean production costs of approximately US$ 2.5–3.0/kg for *L. vannamei*, compared to US$ 3.0–4.0/kg for *P. monodon* culture. *Monodon* culture costs are primarily higher due to increased protein requirements in the feed (FAO, 2008).

1.10 Nutritional Requirement

The nutrition of farmed penaieds is primarily provided by two sources: (1) feed and (2) natural biofloc. In the past, shrimp farmers have looked to feed companies to provide them a feed that is cost effective and still meets minimal nutritional needs. There has been research conducted over the past twenty years in an attempt to determine efficient formulas (Lim *et al*., 1997; Davis *et al*., 2002a; Samocha *et al*., 2004; Cordova-Murueta and Garcia-Carreno, 2002; Cruz-Suárez *et al*., 2007; Otubusin *et al*., 2009). Protein accounts for the majority of shrimp feed content and expense. However, a proper balance of the protein, lipids, amino acids, and vitamins is required to maintain growth and resilience to disease (González-Félix and Perez-Velazquez, 2002).

1.10.1 Protein Nutrition

Protein is a critical ingredient in determining survival, growth response, and cost of production (Lim *et al*., 1997; Kureshy and Davis, 2000; Davis, 2005). Optimal dietary protein levels in penaeid shrimps, measured as growth response, vary from 50–55% in *Penaeus japonicus*, to 40–46% in *Penaeus monodon*, and over 30–50% in *Litopenaeus vannamei* (Cousin *et al*., 1993). For juvenile *L. vannamei*, Colvin and Brand (1977) reported less than 30% to be the protein requirement while Kureshy and Davis (2000) found a maximum protein requirement at 32% for juveniles and sub-adults. Dietary protein levels ranging from 30 to 60% have been recommended for

various species of marine shrimp (Akiyama *et al*., 1992; Davis, 2005). Sources of fishmeal have traditionally included fishmeal, squid, crab, and bivalves (Guillaume *et al*., 1999). However, aquaculture facilities have been able to utilize lower protein feeds (25%) if sufficient natural production and biofloc is available (Hopkins *et al*., 1995; Tacon and Barg, 1998). As seen in Table 8, recommended protein levels required in feed decreases as the shrimp grows (Treece and Fox, 1993).

Table 8. Recommended protein levels for different sizes of penaied shrimp (Treece and Fox, 1993).

Shrimp size (g)	Recommended Feed Protein Level (%)
0.002-0.25	**50**
0.25-1.0	**45**
1.0-3.0	**40**
>3.0	**35**

The popularity of *L. vannamei* as a farmed species has drawn a great deal of work on the dietary requirements of the species (Andrews *et al.*, 1972; Jauncey, 1982; Hopkins *et al*., 1994; McIntosh *et al.*, 2000; McIntosh *et al.*, 2001; Tacon *et al*., 2002; Patnaik *et al.*, 2006). As with many farmed animals, the before mentioned costs of production is critically linked to the cost of feed. The ability of *L. vannamei* to thrive when grown on lower protein diets ranging from 20-35% greatly reduces the cost and environmental impact of the feed. High protein diets have high nutritive value and palatability but are expensive and not readily available (Lim and Persyn, 1989). Consequently, as the cost of fish meal increased in the past few years, more research into finding a replacement or a partial substitute for the fish meal from diets has occurred (Davis and Arnold, 2000; Samocha *et al.*, 2004). Research has been conducted using soybean meal as a replacement or partial substitute has been shown to be either a success or a failure (Lim and Dominy, 1990; Samocha *et al.*, 2004; Alvarez *et al*., 2007) and more studies are ongoing. Generally it is an accepted now that soybean meal can replace a large amount of fish meal without a loss in growth or survival rates (Samocha *et al.*, 2004; Alvarez *et al*., 2007).

When determining the optimum protein concentration, the carbohydrate content needs to be considered as an increase of carbohydrates allows for a decrease of protein without a marked decrease in growth (Guillaume *et al*., 1999). For juvenile *L. vannamei*, a feed containing 5% carbohydrates requires the protein content to be near 55%. However, when the carbohydrate percentage is increased to 25%, no marked decrease is found if the protein content is reduced to 45% (Guillaume *et al*., 1999).

1.10.2 Lipid Nutrition

Penaeid shrimp require dietary lipids for a variety of metabolic functions. Cholesterol, phospholipids, and essential fatty acids are among the most important lipids to promote growth, survival, and normal metabolic function (Guillaume *et al*., 1997; González-Félix and Perez-Velazquez, 2002; Davis, 2005; Patnaik *et al*., 2006). Research carried out by González-Félix and Perez-Velazquez (2002) also found that sterols and carotenoids could impact growth. Through experimentation, it has been demonstrated that shrimp have limited ability to synthesize de novo the n-6 and n-3 families of fatty acids (FA). To a lesser degree, polyunsaturated linoleic (18:2n-6, LOA) and linolenic (18:3n-3, LNA) acids can be synthesized and shrimp have a limited ability to elongate and desaturate these polyunsaturated fatty acids (PUFA) to highly unsaturated fatty acids (HUFA) (González-Félix and Perez-Velazquez, 2002; Patnaik *et al*., 2006). Table 9 identifies the fatty acids that are considered essential fatty acids (González-Félix and Perez-Velazquez, 2002).

As opposed to protein and carbohydrate content, cholesterol and other lipids are only needed at low concentrations. Optimal concentration for cholesterol is close to 1% and total lipids at 8% (Guillaume *et al*., 1999). Increasing the concentration to 16.5% has no effect on growth or survival (Guillaume *et al*., 1999).

Table 9. Essential Fatty Acids (González-Félix and Perez-Velazquez, 2002)

Polyunsaturated Fatty Acids (PUFA)	
linoleic	18:2n-6
linolenic	18:3n-3
Highly Unsaturated Fatty Acids	
arachidonic	20:4n-6
eicosapentaenoic	20:5n-3
docosahexaenoic	22:6n-3

1.10.3 Amino Acids

Determining the optimal dietary amino acid profile is important if shrimp diets are to utilize alternative, less expensive protein sources such as casein (Deshimaru, 1982) and soybean meal (Akiyama, 1988; Samocha *et al.*, 2004). It has been suggested that the amino acid composition required in the feed can be calculated as the levels in feed should be similar to the amino acid levels free in tissue following a feeding (Deshimaru and Shigeno, 1972; Wilson, 1994; Mente *et al.*, 2002) and using whole body analysis (Sudaryono *et al.*, 1996; Penaflorida, 1989). Based on the hypothesis that concentration of an individual free amino acid will remain low until its requirement is met, often researchers have used changes in tissue free amino acid levels to determine amino acid requirements (Wilson, 1994).

Table 10. Essential amino acids ration of whole body juvenile and adult *P. monodon* (Penaflorida[1], 1989; Sudaryon[2] *et al.*, 1996)

Essential Amino Acid	Juvenile[1] (µmol/g)	Adult[2] (µmol/g)
Methionine[a]	**7.61**	**7.40**
Threonine	**5.55**	**7.55**
Valine	**9.43**	**9.85**
Isoleucine	**8.11**	**8.49**
Leucine	**15.44**	**14.60**
Phenylalanine[b]	**16.79**	**15.54**
Lysine	**15.47**	**14.46**
Histidine	**4.59**	**4.74**
Arginine	**17.00**	**15.25**

[a] Methionine plus cystine
[b] Phenylalanine plus tyrosine

Penaflorida (1989) evaluated optimal protein levels using essential amino acid index and determined optimal levels for *P. monodon* juveniles (Table 10). Adult levels were determined by using whole body analysis (Sudaryon *et al*., 1996). The analyses by Penaflorida (1989) for juveniles and Sudaryon (1996) for adults provided information that was helpful for feed formulation as the levels of essential amino acids were different for juveniles than adults (Table 10). As for *L. vannamei*, the quantitative requirements of essential amino acids have not been explored in great detail except for the necessary levels of arginine (Chen *et al*., 1992).

1.10.4 Vitamin and Mineral Nutrition

Vitamins, both water soluble and lipid soluble, are essential for crustaceans and their absence will result in a rapid death of the organism (Guillaume *et al*., 1999). To understand vitamin and mineral minimum requirements, studies have been conducted to identify the individual roles for each and their impact if removed or reduced in diets (Davis *et al.*, 1992). Once the minimum is established, it is important to minimize the loss due to leaching into the water (Cuzon *et al*., 2004). Feed manufactures will typically over fortify the feeds to reduce the effect of leaching into water (Cuzon *et* al., 2004). Vitamin C, for example, is rapidly lost to leaching and has promoted research to find a more stable form such as ascorbyl phosphate (Guillaume *et al*., 1999). Prior to the advent of the stable form (stay-C), high doses of vitamin C were added (up to 10,000 mg/kg diet) for optimal growth. Feeds now include 50-100 mg/kg diet of stay-C (Cuzon *et al*., 2004). Vitamin C has been shown to improve survival when levels are above 30 mg/kg diet (He and Lawrence, 1993a). Among liposoluble vitamins (A, D, E, and K), vitamin E has been explored the most extensively and vitamin E free diets resulted in the lowest survival as compared to vitamin A, D, and K free diets (He and Lawrence, 1993b).

1.11 Factors Affecting Growth Response

1.11.1 Abiotic Factors

Since the majority of nutrient-requirement studies involve measuring a growth

response, particular attention must be taken to control abiotic factors. Stress impacts the ingestion rates and behavioral patterns of the shrimp. Primary abiotic factors that can be controlled are dissolved oxygen, temperature, and salinity. Dissolved oxygen (DO) is a limiting factor which reduces growth through its affect on metabolism (Rosas *et al*., 1998). *L. vannamei* appears to be more tolerant to reduced DO than other cultured shrimp species; however, DO concentrations should be maintained above 2 mg L^{-1} to avoid significant reductions in growth (Seidman and Lawrence, 1985; Rosas *et al*., 1998).

Temperature has been considered the most important modifier of energy flow and subsequent growth of an organism (Brett, 1979; Handeland *et al.*, 2008). Optimum temperature for *L. vannamei* growth appears to decrease as shrimp size increases, producing an optimum temperature >30°C for small shrimp (3.9 g), 30°C for medium shrimp (10.8 g) and 27°C for large shrimp (>16 g) suggesting the importance of uniform stocking weight and predetermined experimental growth ranges (Wyban *et al*., 1995). Juveniles were found to obtain optimum growth between temperatures of 25°C and 35°C with little difference due to salinity as opposed to adult shrimp that grow better between 27-30°C depending on age (Ponce-Palafox *et al.*, 1997).

Natural fluctuations of salinity in the environment expose this species to a wide range of salinities during the juvenile stage. However, sub-adults (postlarval day 40 (PL_{40}) begin to become intolerant of wide changes in salinity (Davis *et al.*, 2002). In general, research has shown that survival, growth, and energy budget are minimally impacted by salinity although susceptibility to inorganic compounds increases as salinity moves toward 2-3 ppt (Boyd and Clay, 2002).

1.11.2 Inorganic Compounds

Inorganic nutrients such as ammonia and nitrite have been shown to reduce growth and survival. The level of theses inorganic compounds in shrimp systems is greatly influenced by stocking density, feed consumption, and feed and water quality

management practices (Velasco *et al*., 1998). Lin and Chen (2001) estimated that the "safety level" for rearing *L. vannamei* juveniles to be 2.44, 3.55, 3.95 mg/l for ammonia-N and 0.12, 0.16, 0.16 mg/l for NH_3-N in 15 ppt, 25 ppt and 35 ppt, respectively. Adult shrimp (> 1 gram) are not tolerant of ammonia at high concentrations above 4 to 5 mg/l (Boyd and Clay, 2002). Safe concentration of nitrite (NO_2-N) levels is 3.8 mg l^{-1} (Chen and Chen, 1990). Nitrate is not harmful to shrimp at concentrations below 50 mg/l (Boyd and Clay, 2002).

1.11.3 Experimental Design (Feed Frequency)

Experimental design also can contribute to differences in growth rates, which can have an affect on the apparent nutrient requirements. Laboratory experimentation can follow a different regime than that which would be considered commercially viable in scaled-up experiments in outdoor tanks or ponds. Laramore (unpublished results) determined that for feed trials designed to determine differences in feed components and not just maximum growth, feeding to cessation once per day was acceptable. However, for intensive growout it was reported by Robertson *et al.* (2008) that *L. vannamei* fed four times during the day had faster growth rates than those fed the same ration over the entire day including the night.

1.11.4 Ingestion and Attractability

The inclusion of non-marine proteins into crustacean diets on ingestion and attractability of the formulated diets must be considered. Both factors have been shown to affect growth (Lawrence and Castille, 1993; Smith *et al*., 2005) and studies have been carried out to evaluate the behavioral response to selected feed attractants and stimulants (Nunes *et al.*, 2006). Smith *et al*. (2005) showed *P. monodon* exhibited significantly greater preference for feeds which contained crustacean or krill meal. Formulating diets with small amounts of chemostimulants might increase ingestion rate and consequently improve growth, survival, and food conversion if the diet formulation was sound (Huang *et al.*, 2003).

1.12 Digestion in *Litopenaeus vannamei*

The gut in *L. vannamei* is basically a simple tube that runs the length of the body from the mouth to the anus at the end of the last somite. Enzyme secretion is limited to the midgut which is comprised of a large number of simple, fragile tubules. Dietary proteins are digested by proteinases such as trypsins and chymotrypins (Lan and Pan, 1993; Chevalier and Wormhoudt, 1998) and these proteinases may be responsible for 40 to 60% of the total protein digestion that occurs in the gut (Tsai *et al*., 1986). The midgut is a major lipid storage organ, mainly storing triglycerides, which are the primary energy source following molting (Birnbaum, 2003). Carbohydrates are digested by alpha-amylase and alpha-glucosidase (Chevalier and Wormhoudt, 1998). Once digested, nutrients are absorbed in the midgut and fecal formation and defecation takes place in the hindgut. This digestive scheme allows *L. vannamei* to be highly effective at digesting protein (Akiyama *et al*., 1992; Aquacop, 1989) even though it lacks pepsin and an acidic stomach.

2. Statement of Purpose and Importance of this Research

2.1 Statement of Purpose of this Research

The research to be presented is aimed at determining more resource efficient feed formulations using a sustainable protein source, specifically algal by-product from biofuel production, in *Litopenaeus vannamei* aquaculture.

2.2 Statement of Importance of this Research

In the long run, the trend toward using non-renewable energy sources is ultimately unsustainable (Christi, 2007). As the demand for fuel increases each year especially in developing nations (Krauss and Bradsher, 2014), a shift toward renewable energy sources may mitigate the unequal balance of supply-and-demand that exists from time to time (Schenk *et al*., 2008). Consequently, scientists are being asked to address and solve the potential energy shortfall. To a great extent, biofuel is currently produced from plant and animal oils, but not from microalgae. Continued research into the feasibility of using microalgae may shift some of the production away from the traditional, less efficient sources (plant and animals oils) toward microalgae (Christi, 2007). Proteins, carbohydrates and natural oils are the three main components of algal biomass. After processing algae for bio-diesel production, substantial amounts of protein and carbohydrate by-product remain. This by-product is promising as a nutrient source.

At present, the by-product remaining, after the oils are removed, has been used to produce a variety of products such as cosmetics and food additives (Sporalore *et al*., 2006). If additional uses for the by-product can be identified and proven experimentally and commercially viable, then it is logical to assume that the sustainability and cost effectiveness of the bio-diesel from algae production model increases.

As previously mentioned, the pace at which global aquaculture production is increasing and finding suitable sources of nutrition can help sustain the growth. Based on the experience I gained working with a commercial shrimp hatchery and grow-out facility, it appears that production costs, including feed, were the primary obstacle preventing the business from being profitable. By considering different methods to reduce feed costs, it became apparent that a unique opportunity exists with respect to the potential use of biofuel-related algal biomass. If a protocol can be developed for the use of biofuel as a primary protein source then it may be possible to optimize production costs, increase the health of the shrimp aquaculture industry, and provide an enhanced outlet for a key waste product from a biofuels process.

The research discussed here, with its focus on more efficient feed formulations using a sustainable protein source for *Litopenaeus vannamei* may not only provide a new protein source for this widespread aquaculture species but also may lead to a model for formulating diets for other commercially important crustaceans.

2.3 Hypothesis 1. Viable Nutritional Source

Feed containing algal biomass, collected after biofuel extraction, can be a partial replacement of marine proteins and a viable nutritional source in shrimp feed.

Inference – The shrimp *Litopenaeus vannamei* will exhibit equal or greater growth and survivability being fed diets formulated with algal biomass, collected after biofuel extraction, as a partial replacement of marine proteins. (H_0)

Experimental Overview – Three algae species, *Chaetoceros calcitrans*, *Nannochloropsis salina*, and *Pavlova sp.* will be collected and processed (removal of lipids) to be used as a replacement component for marine protein (fish meal). The diet will be fed to juvenile *L. vannamei* (PL_{25}) for a period of

six weeks. Growth and final weight data will be collected and compared to a commercially available diet (CONTROL) and a diet formulated with all the ingredients used for the experimental diets without the inclusion of algal biomass (BASAL).

Expected Results – It is expected that the growth will be within 10% of the control diet supporting the hypothesis that algal biomass collected after biofuel production can be a viable nutritional source for the aquaculture of *L. vannamei*.

2.4 Hypothesis 2. High Versus Low Lipid Levels in Algae

The algae used in biofuel production with higher lipid content will be nutritionally inferior, after extraction, to those with less lipid, and presumably higher protein content, when used as a shrimp feed protein source. (H_0)

Inference – If juvenile *L. vannamei* are fed an experimental diet formulated using algal biomass (processed) from high-lipid algae (*Chaetoceros calcitrans*) then the growth will be less than those fed an experimental diet using algal biomass (processed) from low-lipid algae (*Nannochloropsis salina*, and *Pavlova sp.*).

Experimental Overview – Three algae species with differing lipid content, *Chaetoceros calcitrans, Nannochloropsis salina*, and *Pavlova sp.* will be used as a replacement component for marine protein (fish meal) in 18 experimental diets. The diets will be fed to juvenile *L. vannamei* (PL_{25}) for a period of six weeks. Growth and final weight data will be collected and compared among the diets.

Expected Results – Better growth will be exhibited when feeds are formulated with algae having lower lipid levels (*Nannochloropsis salina*, and *Pavlova sp.*) than those having higher lipid levels (*Chaetoceros calcitrans*).

3. Materials and Methods

3.1 Algae Species

Prior to this study, research in the biofuel field has focused on algae that produced high levels of lipids under traditional culture methods and algae that can maximize lipid production when culture conditions are manipulated (i.e., stressed) (NREL, 1998). The time and resources devoted to this endeavor have been considerable (NREL, 1998; Belarbi *et al*., 2000; Christi, 2007). From the over 3,000 species researched by the National Renewable Energy Laboratory (NREL, 1998), three species of algae were chosen for this study as each is commonly used as a nutritional source for *L. vannamei*, their culture temperature threshold was between 22-34ºC, and they can be cultured in a common medium (F/2) (Lavens and Sorgeloos, 1996; Hoff and Snell, 2008).

3.1.1 *Chaetoceros calcitrans* (CCMP1315 – Appendix A)

Table 11. *Chaetoceros calcitrans* - Composition of biomass

Nutrient	% dry biomass
Protein	56.7
Lipids	25.8
Carbohydrate	14.7
Ash	2.80

3.1.2 *Nannochloropsis salina* (CCMP369 – Appendix A)

Table 12. *Nannochloropsis salina* - Composition of biomass

Nutrient	% dry biomass
Protein	58.6
Lipid	14.5
Carbohydrate	20.0
Ash	5.90

3.1.3 *Pavlova sp*. (CCMP459 – Appendix A)

Table 13. *Pavlova sp.* - Composition of biomass

Nutrient	% dry biomass
Protein	51.6
Lipid	19.6
Carbohydrate	22.0 – 24.0
Ash	4.80 - 6.80

3.2 Algae

The initial culture for *Chaetoceros calcitrans* was provided by Earthcare Aquaculture (Clewiston, FL) that used CCMP525 (Provasoli-Guillard National Center for the Culture of Marine Phytoplankton; CCMP) as the inoculant. *Nannochloropsis salina* (Product Nanno 3600: strain CCMP369) and *Pavlova sp*. (Product Pavlova 1800: Strain CCMP459) were purchased from Reed Mariculture (Cambell, California). Each of the cultures was grown under typical culture methods (Hoff and Snell, 2008) and methods to increase lipid content were not employed (Sheehan *et al*., 1998; Lundquist *et al*., 2010; Collet *et al*, 2014). These cultures are microalgae concentrates that are used as larviculture feeds. The algae were processed using the protocol outlined in 3.8 below.

3.3 Algae Cultivation Methods

Standard algae cultivation techniques were used as previously described (Guillard, 1973; Lavens and Sorgeloos, 1996; Hoff and Snell, 2008). Starting with 20 ml test tubes, the culture was gradually transferred to larger containers until a final volume suitable for a 700 L tank was obtained. All growout containers were rinsed with muriatic acid followed by sterilized water (Lavens and Sorgeloos, 1996; Hoff and Snell, 2008). Algal growout was carried out in 19 L carboys and 700 L tanks. Lighting was provided by "Cool White™" fluorescent bulbs that emit 2,800 lumens at the 400-700 nm wavelengths. Batch culture standards starting from 20 ml test tubes upwards to 19 L carboys (bottom surface area 0.09 m^2) were employed. After 7 days, one 19 L carboy was used to inoculate 300 liters of filtered water in a 700 L

transparent tank (Aquatic Ecosystems, Apopka, FL, Part Number T30). The 700 L tanks had a flat bottom, were 1.5 m tall, and had an overall diameter of 0.76 m.

Commercially available mix of Guillard's F/2 was used as the culture medium (Model F2A1 & F2B1, Aquatic Ecosystems, Apopka, FL, USA; Appendixes B-D). For the culturing of *Chaetoceros calcitrans,* sodium metasilicate was added at 1 gram per 75.6 liters (20 gallons) of culture water (Hoff and Snell, 2008).

3.4 Cleaning Culture Equipment

During culture, contamination with bacteria, protozoa, or undesired species of algae may occur. The culture medium consisting of culture water and nutrients, supplied air, culture vessel, or the starter culture that was used could be contaminated with non-targeted algae strains either from improper handling or incomplete cleaning of vessels (Lavens and Sorgeloos, 1996). To reduce the likelihood of contamination and cross-contamination between algal species, all culture equipment was routinely cleaned. All glassware was cleaned and sterilized using dilute muriatic acid prior to use. The culture vessels were covered after sterilization with paraffin paper (Parafilm®). To prevent contamination within the vessels, sterile surgical gauze was used to plug sterilized flasks (Hoff and Snell, 2008). Laboratory utensils, feed trays, measuring cups, and additional items were washed using a laboratory cleaner (Alconox®) diluted at 1 tablespoon per 3.7 liters.

3.5 Water Source

Well water at the Nova Southeastern University Halmos College of Natural Sciences and Oceanography was pumped from a shallow well using a 1.5-HP High Flow Pool Pump into a 4500 L low-density polyethylene open tank and circulated using a 1/3-HP sump pump to reduce hydrogen sulfide content. To pre-treat the water before use in algal culture, the water was filtered through a filter vessel with a 50-micron bag filter (Aquatic Ecosystems, FV1 and VB50) using a 94 L/min pump as it was pumped into indoor 700 L transparent tanks (Aquatic Ecosystems, Apopka, FL, Part Number

T30; Appendix G). Chlorine disinfection of culture water was accomplished by adding 150 mL of liquid household bleach to the initial 300 L volume. The dosage rate was 0.5 mL bleach per liter of water (Hoff and Snell, 2008). After 24 hours, any residual chlorine was removed using sodium thiosulfate (Aquatic Ecosystems, Product ST1A, Apopka, FL) at a dosage of 1.051 g per 1 ppm of Chlorine in the 300 L cylinders. Prior to addition to the tank, the sodium thiosulfate was dissolved in 100 mL of water taken from the tank using a magnetic stir bar and stirrer. Residual chlorine was detected using a commercial pool test indicator kit and adding sodium thiosulfate as necessary.

3.6 Culture Water Sterilization

Sterilization of culture water for initial inoculates was additionally treated by microwave sterilization (Hoff and Snell, 2008). Each vessel was then covered with sterile gauze for 24 hours and the water allowed to return to room temperature.

3.7 Algae Harvesting

Algae were harvested by pumping the culture water from the 700 L indoor culture tank into a 5-micron filter sock (Aquatic Ecosystems, FVB5) using a 15.8 L/min pump (Aquatic Ecosystems, MD32 Mag Drive Pump) through vinyl reinforced clear tubing. The filter sock was hung inside the 700 L culture vessel and the culture water was recycled back to the original vessel. The pump continued to run until algal biomass inside the sock reached a point that culture water overflowed the sock. The pump was disengaged and the sock taken to the drying aquarium where the algal biomass was removed for further preparation per Section 3.8.

3.8 Oil Extraction and By-product Preparation

Although several methods to remove lipids from the harvested algae have been developed (see 1.6.4 Harvesting and Extraction Methods), this project used “expression” to separate the recoverable lipids, which could be used for biofuel

production, from the algae. The algal biomass was prepared by concentrating the algae using a filter sock (per Section 3.7) and then transferred to a 10 gallon drying aquarium that had two 100-watt bulbs with reflectors mounted above. The bulbs aided the drying process with radiant heat. After 1 hour in the aquarium, algae and water separated into two layers. The supernatant layer, essentially water, was removed by pipette to accelerate the drying process. The drying process lasted for 12-16 hours or until the algae had dried sufficiently to allow "expression" of the lipids by pressure (Appendix E). Pressure was applied by using a 6-ton A-Frame Hydraulic Bench Shop Press (Harbor Freight, Item #1666). The liquid fraction was removed and discarded. The remaining product, i.e., the algal biomass, was collected and refrigerated until used for feed preparation. The product did contain residual some residual moisture as reported in the lab analysis. A 15 gram sample of each pressed product was collected and sent to a commercial laboratory for proximate analysis (see 3.14 Nutritional Analysis of Algal Feed Component).

3.9 Shrimp Source

Postlarval shrimp were obtained from Earthcare Aquaculture (Clewiston, Florida, www.EarthCareAquaculture.com). The postlarvae were collected using dip nets from an indoor tank and transferred to a 19 L plastic bag filled with culture water from the same indoor tank, following the recommended procedures by De Boeck (1990). The bag was then placed inside of a 38 L thermal cooler. The bag was half-full of water and shrimp and half-full of air. As it is essential to maintain adequate oxygen for respiration during transport, pure oxygen was added. First, the air hose was placed inside the bag and the atmospheric air was pressed out of the bag. The oxygen regulator was opened slowly until the bag expanded and filled. Then the bag was sealed closed using several rubber bands to ensure closure and as an added precaution, the first bag was placed inside of another bag (De Boeck, 1990). The animals were transferred to a laboratory at Nova Southeastern University Halmos College of Natural Sciences and Oceanography, Fort Lauderdale, Florida, where they were placed in a two 38 L closed tank system for three days in accordance with the

procedures described by Garza de Yta *et al.* (2004). This nursery period was used to ensure that shrimp were acclimated to the conditions present in the laboratory before the feed trials began. The stocking density of the nursery tank was 15 postlarvae per liter. A crumbled commercial postlarval feed (Cargill Shrimp Starter 3507: 35% protein, 7% fat) was presented twice per day and light regime was 12:12 light:dark. The stocking postlarvae were hand sorted for uniform size upon initiation of the feed trials.

3.10 Feed Formulation Strategy

In the laboratory, nineteen diets were formulated. For eighteen diets, varying levels of marine protein (60, 80, and 100%) were replaced with either dried algae or dried algae that had been pressed to simulate lipid expression for biofuel production (per Section 3.8)(Table 14). A single diet, with 0% fish meal replacement and known from this point forward as BASAL diet, was formulated with all of the ingredients but without adding any algae paste. This diet was included to serve as a benchmark to verify the ingredients used would meet the minimal requirements for growth and survival rate of *L. vannamei* (Table 14) as it is expected that a diet with fish meal as the primary protein would perform well. Ingredients were weighed using a compact balance (i101 iBalance Generation 3, Capacity 100 g; Division 0.005 g) that was calibrated with a Troemner Calibration Precision Weight Set (Troemner, Thorofare, NJ). Dry ingredients were mixed in a bowl and then added to a food mixer. Menhaden fish oil was added into the bowl and mixed on low speed for five minutes until homogenized in the food mixer. During the mixing, approximately 50 mL of hot water was blended in to attain an optimal consistency for pelleting. As the quantity of feed being produced was not sufficient to pass through a pelleting machine, the mixture was extruded through a 15 mL plastic syringe. The extruded strands were placed on an elevated drying rack and dried at 80°F. After drying (approximately 45 minutes) the feeds were hand crumbled (Appendix F). Each diet was refrigerated throughout the trial and removed only when daily rations were needed.

Table 14. Ingredient composition of experimental diets (g/100 g dry weight) fed to *L. vannamei* for 6 weeks under controlled conditions

	Percent Fish Meal Replacement			
Ingredients	**0**	**60**	**80**	**100**
Algae paste	0.00	23.60	31.40	39.30
Menhaden Fish Meal[a]	30.00	12.00	6.00	0.00
Menhaden Fish Oil[b]	4.10	4.10	4.10	4.10
Soybean Meal	17.70	17.70	17.70	17.70
Wheat gluten	4.00	4.00	4.00	4.00
Wheat starch	35.90	29.40	27.50	25.30
Alginate	5.00	5.00	5.00	5.00
Trace mineral premix[e]	0.50	0.50	0.50	0.50
Vitamin premix[f]	2.00	2.00	2.00	2.00
Vitamin C[g]	0.10	0.10	0.10	0.10
Calcium phosphate[h]	0.20	1.10	1.20	1.50
Soy lecithin[i]	0.50	0.50	0.50	0.50
Total	**100.00**	**100.00**	**100.00**	**100.00**

[a] Special Select™, Zapata Protein USA, Randeville, LA, USA.
[b] Omega Protein, Reedville, VA, USA.
[c] United States Biochemical, Cleveland, OH, USA.
[d] Industrial Grain Products, Lubbock, TX, USA.
[e] Mineral Premix (see Appendix C)
[f] Vitamin Premix (see Appendix D)
[g] Stay C®, Roche Vitamins, Parsippany, NJ, USA.
[h] Cefkaphos®, primarily monobasic calcium phosphate), BASF, Mount Olive, NJ, USA.
[i] Aqualipid 95, Central Soya Chemurgy Division, Fort Wayne, IN, USA.

Each diet was assigned a label to identify the algal species (i.e., Chaeto to represent *Chaetoceros calcitrans*; Nanno to represent *Nannochloropsis salina*; Pavlo to represent *Pavlova sp.*) and the percentage of fishmeal replaced (60, 80, or 100%) (Table 15). For example, the label *Chaeto*60L identifies that *Chaetoceros calcitrans* was the algae species used, that 60% of the fish meal was replaced, and that the algae were dried only (the L suffix indicating the absence of lipids). *Chaeto*60 identifies that *Chaetoceros calcitrans* was the algae species used, that 60% of the fish meal was replaced, and that the algae were dried and pressed.

Table 15. Matrix of % Fish Meal Replacement Assignment

	Lipids Removed	% Fish Meal Replacement		
		60	80	100
Chaetoceros calcitrans	Y	Chaet60L	Chaet80L	Chaet100L
	N	Chaet60	Chaet80	Chaet100
Nannochloropsis salina	Y	Nanno60L	Nanno80L	Nanno100L
	N	Nanno60	Nanno80	Nanno100
Pavlova sp.	Y	Pavlo60L	Pavlo80L	Pavlo100L
	N	Pavlo60	Pavlo80	Pavlo100

Table 16 provides the proximate protein composition (%) for the nineteen diets formulated in the laboratory and the one commercially available control diet (CONTROL). The CONTROL diet was included as the benchmark to compare growth and survival for this study. Shrimp were randomly assigned to one of the twenty dietary treatments (Table 17), with seven replicates per treatment.

Table 16. Proximate protein composition (%) for formulated and control diets

	Diet Designation	**Fish Meal Substitution (%)**	**Algal Meal Preparation Dried (d) or Dried & Pressed (dp)**	**Overall Crude Protein in Diet (%)**
Chaetoceros calcitrans	Chaet60	60	d	34
	Chaet60L	60	dp	27
	Chaet80	80	d	35
	Chaet80L	80	dp	26
	Chaet100	100	d	36
	Chaet100L	100	dp	24
Nannochloropsis salina	Nanno60	60	d	34
	Nanno60L	60	dp	24
	Nanno80	80	d	35
	Nanno80L	80	dp	22
	Nanno100	100	d	36
	Nanno100L	100	dp	20
Pavlova sp.	Pavlo60	60	d	34
	Pavlo60L	60	dp	24
	Pavlo80	80	d	35
	Pavlo80L	80	dp	22
	Pavlo100	100	d	36
	Pavlo100L	100	dp	20
	Control	0	n/a	35
	Basal	0	n/a	32

3.11 Feed Trials

The twenty dietary treatments (Table 17; Figure 9) were randomly assigned to shrimp in seven replicates per treatment using a random assignment calculator. Postlarval shrimp were placed in 355 mL Styrofoam cups filled with 200 mL of 32 ppt seawater, one shrimp per cup. Cups were filled with prepared seawater (see 3.5) from Nova Southeastern University Halmos College of Natural Sciences and Oceanography well (salinity 32-35‰) (Appendix G). Water temperature was maintained at 28 ± 2°C by using an electric room heater with a thermostat and a fan to circulate the air throughout the laboratory. To maintain optimal test conditions, 90% of the water in the cups was exchanged per day. Additionally, once per week, all of the water in the vessel was exchanged on the day weights for each animal were taken. Total ammonium-N and nitrite-N were measured weekly using a saltwater test kit (API, Model 401M). Nitrate was not measured. The daily water exchange rate prevented the buildup of ammonia and nitrite as pollutants. These pollutants typically result from the excretion of cultured animals and the mineralization of organic detritus such as unconsumed food and feces in this study (Lin and Chen, 2003). *Nitrobacter*, the nitrogen fixing bacteria from nitrite to nitrate, would not have sufficient time to process as it can take up to three weeks of high ammonia and nitrite to establish an active nitrifying population (Avnimelech *et al*., 1986). A 12-h light and 12-h dark photoperiod was maintained throughout the experiment using a mechanical timer.

Table 17. Assignment of random grid position to each treatment

Feed Label	Treatment	Random Grid Position
CHAET60	1	4
CHAET60L	2	19
CHAET80	3	6
CHAET80L	4	18
CHAET100	5	10
CHAET100L	6	9
NANNO60	7	15
NANNO60L	8	13
NANNO80	9	20
NANNO80L	10	11
NANNO100	11	8
NANNO100L	12	16
PAVLO60	13	3
PAVLO60L	14	5
PAVLO80	15	12
PAVLO80L	16	7
PAVLO100	17	2
PAVLO100L	18	17
CONTROL	19	1
BASAL	20	14

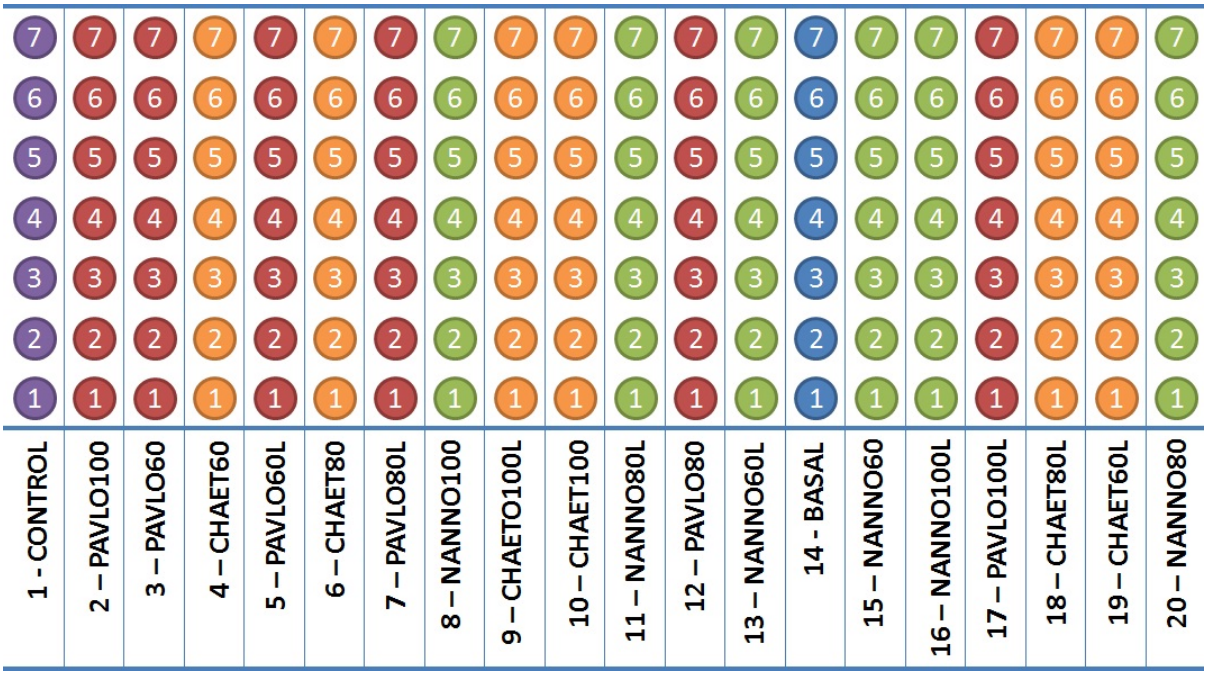

Figure 9. Position of each treatment

At 4 weeks, all treatment vessels were replaced with new treatment vessels.

3.12 Weighing of Animals

Weights were recorded weekly using a Denver Instrument Company Analytical Lab Balance (Serial Number B041948). Prior to each weighing session, the balance was calibrated using its internal calibration system and verified using a Troemner Calibration Precision Weight Set (Troemner, Thorofare, NJ). The verification was performed after each of the seven treatments was weighted. The threshold for miscalibration was 0.05 g. If it was determined that the balance was miscalibrated, all animals from that treatment set would be reweighed. Each replicate was brought to the weighing bench and inverted into an aquarium net catching the animal but allowing the water and waste products to be collected in a 19 L container. The animal was then placed on a paper towel to allow maximum drying without harming the animal. Next, the animal was placed on the weighing boat (Fisherbrand® Pour-Boat Weighing Dish, 3.5" x 5.25" x 1", Cat. No. 02-204-1B) and the doors of the balance were closed. During the few seconds that it took for the balance to come to rest, the treatment vessel was refilled with 200 mL of filtered seawater (see 3.5). Once the balance came to rest, the value was recorded and the doors to the balance were opened. The animal was again returned to its treatment vessel. This process was repeated for all replicates.

3.13 Data Analysis

The method of data collection was considered and the following three measures were taken to minimize errors during the trials. First, all weights were taken with the same balance (see 3.12). Second, the assignment of treatments was random. Third, in an effort to improve precision and accuracy, there were 7 replicates for each treatment.

In the first ANOVA model, the between subject factor was all the species and the within subject factor was the different time periods in weeks. This model was tested

to determine whether the BASAL and CONTROL diets differed from the diets that replaced fish meal with algae.

From the second to the fourth (last) ANOVA model, the within subject factor was the different time periods in weeks; and the between subject factors were experimental species (CHAETO, NANNO, and PAVLO in the second model), Level of the fishmeal replacement (60%, 80%, and 100% in the third model), and the presence of Lipid in the fourth model. These three ANOVA models were tested to examine whether any of the between subject factors produced a different pattern of growth over time.

Prior to running all the ANOVA models, assumption of homogeneity of covariance was checked by the Box's test of equality of covariance, and this assumption was met by the data except for the model-3. And, the assumption of homogeneity of variance was checked by the Leven's test of equality of error variance, and this assumption did not met in 3 cases out of 28 cases (7 weeks × 4 ANOVA models). Also, the assumption of sphericity was tested for all the models, however, this assumption was found not to be met ($p < .05$) and the associated epsilon values for all the four models were found smaller than .75. As a rule of thumb (epsilon < .75), Greenouse-Geisser correction method was considered to adjust the degrees of freedom with a view to address the violation of the assumption of sphericity. Furthermore, using Bonferroni methods, post-hoc tests were also observed for all ANOVA models.

All statistical analyses were conducted using the Generalized Linear Model (GLM) module in Statistical Package for Social Science (SPSS) version 22 (IBM SPSS Statistics for Windows, Version 22.0, Released in 2013, Armonk, NY).

3.14 Nutritional Analysis of Algal Feed Component

Samples of the algae paste (i.e., absence of lipids) (15 g) was sent to the New Jersey Feed Laboratory (Trenton, NJ) for proximate analysis. Standardized chemical analysis methods as provided by the Official Methods of Analysis (AOAC, 2012)

were used to determine the levels of total crude protein (Association of Official Analytical Chemists (AOAC) 990.03), total crude fat (fats, oils, pigments, and other fat soluble substances; AOAC 920.39), fiber (AOAC 978.10), ash (AOAC 942.05), and moisture (AOAC 930.15) of each of the algae.

3.15 Feeding Protocol

Feeds were offered by hand to apparent satiation once per day, at 1200 hrs and uneaten food was removed by pipette. Apparent satiation was determined when each animal ceased feeding on the offered pellet in each vessel. Each vessel was fed in the same order to preserve the number of hours between feedings. Typically, it would take four hours to complete feeding the first replicate to the last replicate. Prior to the daily feeding, feces and exoskeletons were removed from the tanks and discarded. Any dead animals were removed from the treatment but the cup was kept in the matrix as a placeholder to preserve the location of each treatment vessel throughout the entire experiment.

3.16 Feed Attractability, Palatability, Diet Leaching

During a preliminary trial, it was observed that the shrimp did not refuse any of the feed provided. This process was quantified by observing the shrimp and monitoring each until the animal began to feed on the pellet. In all cases, this began within one to two minutes of the pellet's introduction to the container. In trials carried out by Nunes *et al.* (2006) to test feed ingredients, a single animal was placed in a Y-maze measuring 1.3 m x 0.3 m x 0.4 m (length x width x height). If the animal did not detect the location of the food within, the animal was replaced by another. Some ingredients were found to be rejected by the animal as was observed for the control pellets made of only gelatin without any attractants, e.g., condensed fish soluble protein (Nunes *et al*., 2006).

The results of the preliminary trial demonstrated that there was not a need for additional trials when using the current experimental setup, which included small vessels with static water exchange. It was not necessary to calculate diet leaching for this experiment due to the short time from when the feed entered the water to when the shrimp began to feed (one to two minutes).

3.17 Harvest

After 6 weeks, shrimp were harvested, blotted dry, and weighed individually by treatment vessel per the protocol outlined in Section 3.12. The final weight of each animal was recorded and total survival calculated for each treatment (Table 19).

3.18 *In vivo* Experiments

Water quality

Ammonia-N, nitrite, and nitrate did not confound the experimental results and the recommended levels from literature were not exceeded as 180 mL of filtered seawater were replaced daily (Section 3.11). As previously mentioned, Lin and Chen (2001) estimated that the "safety level" for rearing *L. vannamei* juveniles to be 3.95 mg/l for ammonia-N and 0.16 mg/l for NH_3-N in 35 ppt water. Adult shrimp (> 1 gram) are not tolerant of ammonia at high concentrations above 4 to 5 mg/l (Boyd and Clay, 2002). Safe concentration of nitrite (NO_2-N) is 3.8 mg/l (Chen and Chen, 1990). Ammonia-N and nitrite levels were maintained below the recommended levels (Chen and Chen, 1990; Chen and Lin, 1991; Lin and Chen, 2001; de Lourdes Cobo *et al*., 2014). This was confirmed by testing (Section 3.11). Nitrate levels were not measured due to the short residence time of the water in the cups which prevented any nitrate build-up.

Values obtained during the experiment were below recommended levels, which suggest shrimp were maintained under optimal water quality parameters, relative to these compounds for the duration of the trial.

4. RESULTS

Four statements summarize the results of this study:

- Growth and survival of *Litopenaeus vannamei* larvae were within industry guidelines.
- Protein levels for *Chaetoceros calcitrans*, *Nannochloropsis salina*, and *Pavlova sp.* was 56.7, 58.6, and 51.6 % dry biomass respectively. Following the partial extraction of lipids, the protein levels dropped to 29.0, 16.9, and 16.9.
- Growth rates for the individual species were significantly lower than growth rates for the controls.
- The analysis of differences between species, levels, and the absence or presence of lipids indicated that significantly higher growth rates were found for the conditions in which replacement level equaled 60 rather than 100.

4.1 Evaluation of Experimental Diets

The preparation of the diets using the algal paste after lipid extraction caused a marked decrease in crude protein as compared to dried algal biomass. The initial protein levels for *Chaetoceros calcitrans*, *Nannochloropsis salina*, and *Pavlova sp.* was 56.7, 58.6, and 51.6 % dry biomass respectively. After the partial extraction of lipids, the drying was carried out using recommended practices (Cruz-Suárez *et al*., 2007); however, an elevated amount of ash was identified during analysis which reduced percent protein with a proportionate increase in the percent ash (Table 18).

Table 18. Analysis of algae

Algal Species	Initial Protein Levels (%)	Final Protein Levels (%)
Chaetoceros calcitrans	56.7	29.0
Nannochloropsis salina	58.6	16.9
Pavlova sp.	51.6	16.9

As previously mentioned in 1.10.4, leaching of nutrients can occur when feeds are introduced into the water which will ultimately decrease the total available nutrients

for the animal (Cuzon *et al*., 2004). During this study, it can be assumed that leaching had minimal impact as shrimp began feeding immediately on the feed being offered. Additionally, due to the rapid consumption of the offered diet, even though the amount of water absorption by the formulated diets appeared to be greater than the control diet, the affect would be minimal. A commercial pelleting process would be required if the formulated feeds were going to be used in an open water system.

4.1.1 Growth and Survival of *L. vannamei*

The results of the growth trial at 42 days are presented in Table 19. All treatments concluded the trial with 71% survival or higher. Initial weights for the juvenile *L. vannamei* were 0.0306 ± 0.0011 g. Final weights ranged from 0.1547 to 0.2422 g and were numerically the highest in Chaet60L treatment. Instantaneous growth rate ranged from 9.24 to 17.30% per day.

Each treatment's response over the trial is graphed in Figure 10.

Table 19. Response of postlarval *L. vannamei* to practical diets containing increasing levels of algal biomass with and without lipids replacing fish meal on a percentage basis

Diet Designation	Fish Meal Substitution (%)	Algal Meal Preparation Dried (d) or Dried & Pressed (dp)	Initial Weight (g)	Final Weight (g)	Weight Gain (g)	Growth (% initial weight)	Instantaneous Growth Rate (%)	Survival (%)
Chaet60	60	d	0.0308	0.2157	0.1849	600.32	14.29	71
Chaet60L	60	dp	0.0293	0.2422	0.2129	726.62	17.30	71
Chaet80	80	d	0.0306	0.1911	0.1605	524.51	12.49	86
Chaet80L	80	dp	0.0296	0.1790	0.1494	504.73	12.02	86
Chaet100	100	d	0.0308	0.1896	0.1588	515.58	12.28	100
Chaet100L	100	dp	0.0295	0.1742	0.1447	490.51	11.68	100
Nanno60	60	d	0.0307	0.2252	0.1945	633.55	15.08	86
Nanno60L	60	dp	0.0316	0.2212	0.1896	600.00	14.29	86
Nanno80	80	d	0.0306	0.1822	0.1516	495.42	11.80	86
Nanno80L	80	dp	0.0268	0.1947	0.1679	626.49	14.92	100
Nanno100	100	d	0.0298	0.1783	0.1485	498.32	11.86	86
Nanno100L	100	dp	0.0299	0.1895	0.1596	533.78	12.71	86
Pavlo60	60	d	0.0303	0.2051	0.1748	576.90	13.74	100
Pavlo60L	60	dp	0.0295	0.1996	0.1701	576.61	13.73	86
Pavlo80	80	d	0.0301	0.2340	0.2039	677.41	16.13	86
Pavlo80L	80	dp	0.0310	0.1822	0.1512	487.74	11.61	100
Pavlo100	100	d	0.0294	0.1815	0.1521	517.35	12.32	71
Pavlo100L	100	dp	0.0317	0.1547	0.1230	388.01	9.24	100
Control	0	n/a	0.0330	0.2078	0.1748	529.70	12.61	86
Basal	0	n/a	0.0287	0.2124	0.1837	640.07	15.24	100

Pavlova sp. *Nannochloropsis salina* *Chaetoceros calcitrans*

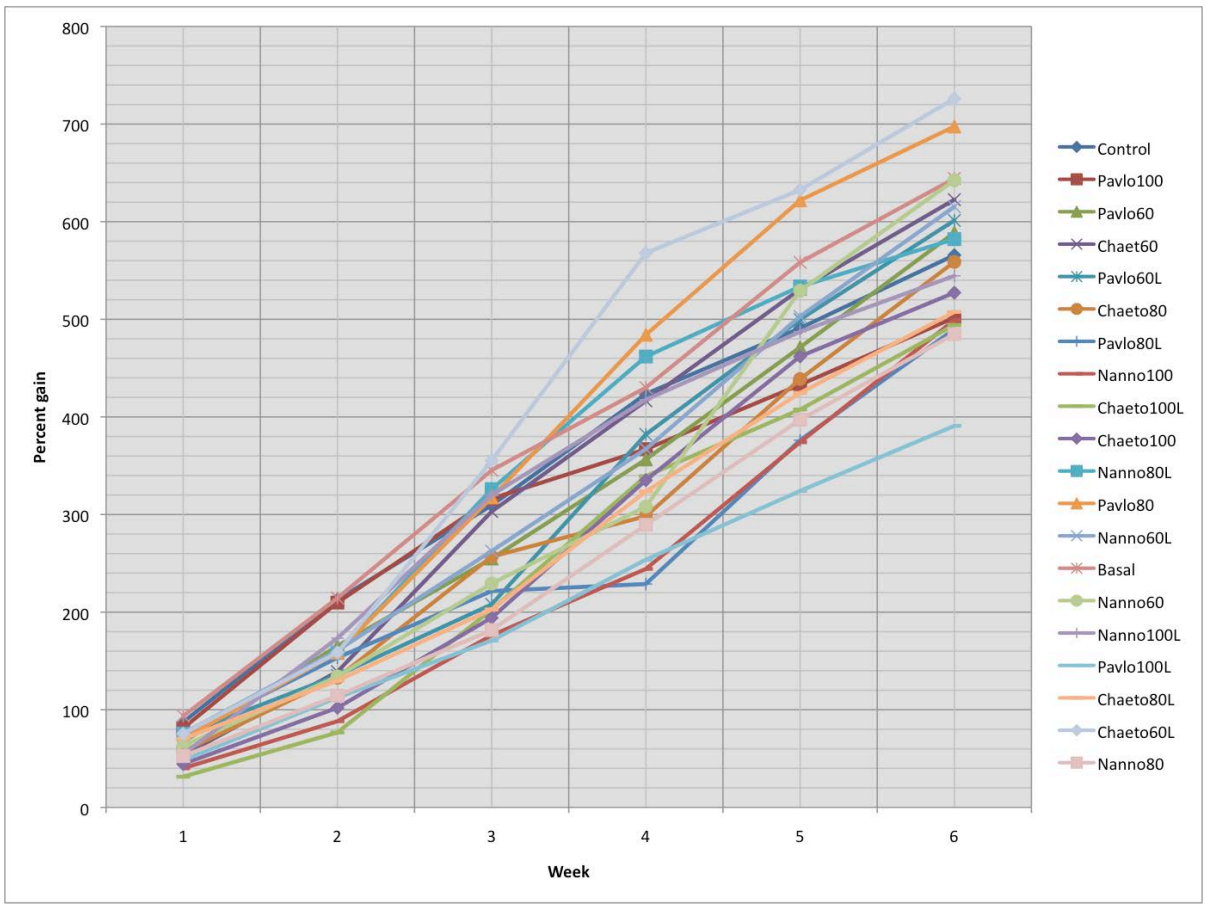

Figure 10. Response by *L. vannamei* to 20 different feed formulations

4.2 Statistical Analyses

The statistical analyses proceeded in two stages through four Split-Plot ANOVA models: Stage-1) comparison of the experimental species with control and basal diets in ANOVA model-1, and Stage-2) examination of differences in growth between species (ANOVA model-2), while also evaluating the effects of the level of fishmeal replacement (60%, 80%, and 100% in ANOVA model-3), and the presence or absence of lipids originating from the algae (ANOVA model-4). Analyses were conducted on replicates that had complete data across all seven weeks; replicates that died prior to the conclusion of the study were not included in the analyses.

4.2.1 Stage-1: Comparison with Control and Basal Diet

The first stage of the analyses examined differences in growth rates between the experimental, BASAL, and CONTROL diets. Effects for the experimental diets were averaged across all levels and the lipid and non-lipid (presence or absence of lipids) conditions. Both the BASAL and CONTROL diets did not contain any algae and are included as a comparison versus the formulated diets with algae. Mean weights are displayed by species and week in Table 20.

Table 20. Analysis of weight by species and week

SPECIES		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
BASAL	Mean	.02874	.05537	.08979	.12683	.15079	.18743	.21243
	n	7	7	7	7	7	7	7
	SD	.002808	.005587	.005020	.007291	.012069	.014764	.017251
CHAETO	Mean	.02984	.04606	.06582	.10359	.13945	.16789	.19602
	n	36	36	36	36	36	36	36
	SD	.003070	.007840	.010815	.025043	.031236	.030388	.031841
CONTROL	Mean	.03235	.06260	.10325	.13515	.16352	.18408	.20783
	n	6	6	6	6	6	6	6
	SD	.007401	.010464	.018872	.015561	.014422	.015478	.021018
NANNO	Mean	.03019	.04789	.07114	.10486	.13450	.17115	.19843
	n	37	37	37	37	37	37	37
	SD	.002490	.005718	.011253	.019828	.024245	.024917	.025067
PAVLO	Mean	.03043	.05081	.07616	.10324	.13254	.16477	.19218
	n	38	38	38	38	38	38	38
	SD	.003051	.005669	.010754	.018027	.026594	.028298	.028983
Total	Mean	.03018	.04939	.07374	.10670	.13766	.16979	.19706
	n	124	124	124	124	124	124	124
	SD	.003211	.007602	.014148	.021766	.027063	.027254	.028017

A repeated measures ANOVA was used to determine if there were significant differences in the average weight, and in the growth in weight. Of particular interest was the test of the time by species interaction. A significant interaction between time and species would indicate that growth rates across time varied between species.

4.2.1.1 Results of the first ANOVA model

The results of the first ANOVA model are presented in Table 21.1 to 21.3, and they do indicate that the main effect of time (in weeks) and the interaction effect between time and species were found to be statistically significant in within subject factor (for the main effect: $F = 1114.46$; $df = 2.21, 262.89$; $p < .001$; for interaction effect: $F = 2.07$; $df = 8.84, 262.89$; $p < .05$). And, the test of between subject results shows that the species are significantly differed from each other ($F = 4102.22$; $df = 4, 119$; $p <$

.001). The post-hoc test results also shows that all the experimental groups (CHAETO, NANNO, and PAVLO) are significantly different from the control group ($p < .05$) in terms of the average growth. However, the experimental groups are not significantly different from the BASAL group ($p > .05$).

Table 21.1 Repeated ANOVA for species – Tests of within-subjects effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Greenhouse-Geisser	1.544	2.209	.699	1114.459	.000
Time * SPECIES	Greenhouse-Geisser	.011	8.836	.001	2.074	.033
Error(Time)	Greenhouse-Geisser	.165	262.858	.001		
a. Computed using alpha = .05						

Table 22.2 Repeated measures of ANOVA for species – Tests of between-subjects effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	5.843	1	5.843	4102.219	.000
SPECIES	.023	4	.006	4.108	.004
Error	.169	119	.001		
a. Computed using alpha = .05					

Table 23.3 Post-hoc Test – Pairwise Comparison

(I) SPECIES	(J) SPECIES	Mean Difference (I-J)	Std. Error	Sig.[b]
CONTROL	CHAETO	.020*	.006	.019
	NANNO	.019*	.006	.036
	PAVLO	.020*	.006	.020
	BASAL	.005	.008	1.000
BASAL	CHAETO	.015	.006	.142
	NANNO	.013	.006	.253
	PAVLO	.014	.006	.151
	CONTROL	-.005	.008	1.000
*. The mean difference is significant at the .05 level.				
b. Adjustment for multiple comparisons: Bonferroni.				

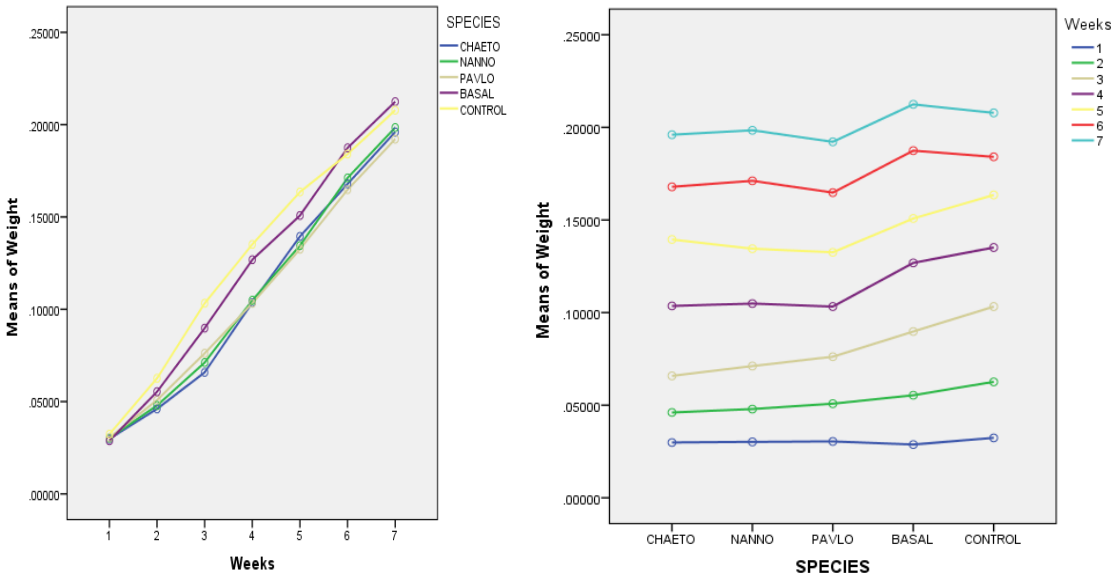

Figure 11. Plot of weight by species over time

The mean weights for each species are plotted in Figure 11. The means of weight represents the average weights of diets for each species regardless the absence or presence of lipids. It can be seen in the graphs that weights increased more quickly for the Control and Basal groups compared to the CHAETO, NANNO, and PAVLO groups towards the end of the treatment.

4.2.2 Stage-2: Analysis of Species, Percent Replacement, and Lipids

The next stage of the analysis examined both the main effect of times (in weeks) and the interaction effects with experimental species only (CHAETO, NANNO, and PAVLO in the second ANOVA model), level of fishmeal replacement (third ANOVA model), and presence or absence of lipids (fourth ANOVA model) on weight. Mean weights for each species have already been shown earlier (see Table 19). Mean weights by percentage of fishmeal replacement (or level) are shown in Table 22, while Table 23 shows mean weights by Lipid/Non-Lipid.

Table 24. Weight by percent replacement and time

Level		w1	w2	w3	w4	w5	w6	w7
60	Mean	.03011	.05049	.07504	.10949	.14654	.18542	.21719
	N	35	35	35	35	35	35	35
	Std. Deviation	.002983	.005359	.008989	.019404	.026260	.023866	.025130
80	Mean	.02994	.05000	.07198	.10532	.13359	.16575	.19358
	N	38	38	38	38	38	38	38
	Std. Deviation	.002733	.006457	.008824	.021653	.030123	.029374	.026328
100	Mean	.03041	.04458	.06668	.09733	.12706	.15394	.17747
	N	38	38	38	38	38	38	38
	Std. Deviation	.002945	.006618	.014670	.020282	.022226	.020428	.019359
Total	Mean	.03016	.04830	.07113	.10390	.13544	.16791	.19551
	N	111	111	111	111	111	111	111
	Std. Deviation	.002867	.006704	.011640	.020929	.027371	.027808	.028594

Table 25. Weight by process (dried vs dried and pressed) and time

Lipid		w1	w2	w3	w4	w5	w6	w7
0 No Lipid	Mean	.03027	.04747	.07116	.10357	.13365	.17126	.20027
	N	54	54	54	54	54	54	54
	Std. Deviation	.002986	.006070	.011942	.019667	.025041	.026467	.026529
1 Lipid	Mean	.03005	.04908	.07111	.10421	.13712	.16474	.19100
	N	57	57	57	57	57	57	57
	Std. Deviation	.002772	.007220	.011454	.022229	.029532	.028895	.029957
Total	Mean	.03016	.04830	.07113	.10390	.13544	.16791	.19551
	N	111	111	111	111	111	111	111
	Std. Deviation	.002867	.006704	.011640	.020929	.027371	.027808	.028594

In this stage of analyses, three different repeated measures ANOVA were utilized to determine whether there were significant differences in weight and in growth rates between species, levels of species, and lipid versus non-lipid variations. These three ANOVA models included one within subject factor (time) and three between subject factors (species, level, and lipid/non-lipid). Of particular interest are the two-way interactions observed with time and the between subject factors. In particular:

- A significant two-way time by species interaction would indicate that growth rates differed between species.
- A significant two-way time by level interaction would indicate that growth rates differed between levels.
- A significant two-way time by lipid/non-lipid interaction would indicate that growth rates differed significantly according to the absence or presence of lipids.

4.2.2.1 Results of the second ANOVA model

In this model the within subject factor was time and the between subject factor was the experimental species only, namely, CHAETO, NANNO, and PAVLO. The results of the second ANOVA model are presented in Table 24.1 and 24.2. The test results indicate that the main effect of time (in weeks) was found to be statistically

significant (F = 1742.11; df = 2.18, 235.93; p < .001) in within subject factor, however, the interaction effect was found not be statistically significant (F = 1.58; df = 4.37, 235.93; p > .05). And, the test of between subject results shows that the growths between the experimental species are not significantly differed from each other (F = .09; df = 2, 108; p > .05). No post-hoc test was observed for this model as the interaction effect was not statistically significant.

Table 264.1 Repeated ANOVA for experimental species – Tests of within-subjects effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Greenhouse-Geisser	2.554	2.184	1.169	1742.107	.000
Time * SPECIES	Greenhouse-Geisser	.005	4.369	.001	1.577	.176
Error(Time)	Greenhouse-Geisser	.158	235.926	.001		
a. Computed using alpha = .05						

Table 274.2 Repeated ANOVA for experimental species – Tests of between-subjects effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	8.971	1	8.971	5940.714	.000
SPECIES	.000	2	.000	.091	.913
Error	.163	108	.002		
a. Computed using alpha = .05					

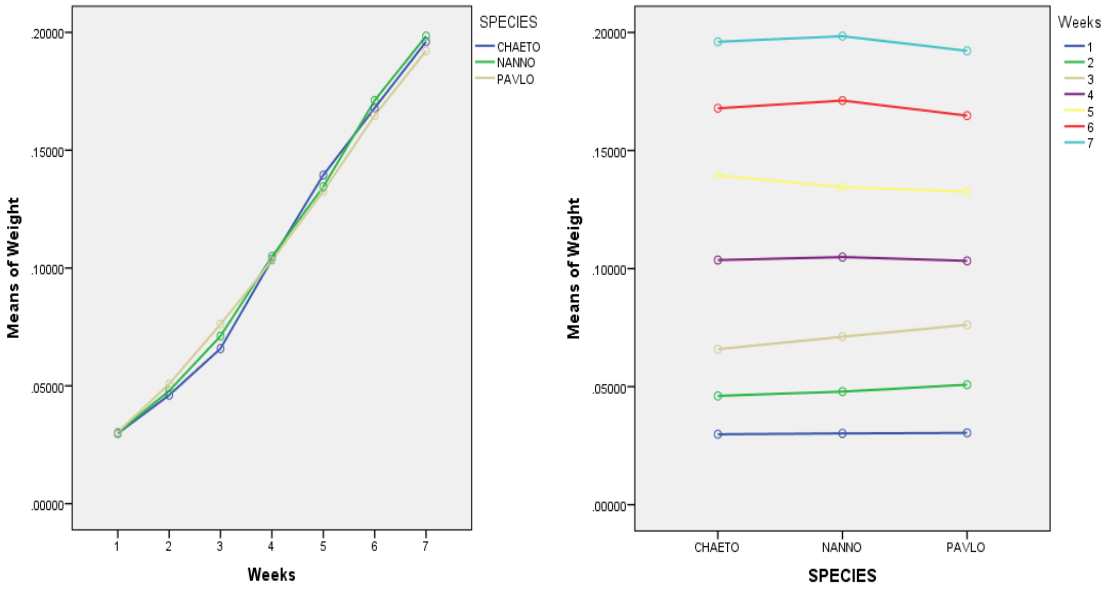

Figure 12. Plot of weight by experimental species over time

The mean weights for experimental species are plotted in Figure 12. The means of weight represents the average weights of diets for each species. It can be seen in the graphs that comparatively the growth rate of PALVO started increasing from second to third week while the growth of NANNO was higher in the sixth and seventh week, however, there was no significant increasing growth between the species was observed.

4.2.2.2 Results of the third ANOVA model

In this model the within subject factor was time and the between subject factor was the level of the fishmeal replacement, namely, 60%, 80%, and 100%. The results of the third ANOVA model are presented in Table 25.1 to 25.3. The test results indicate that both the main effect of time (in weeks) as well as the interaction effect between time and levels of fishmeal replacement were found to be statistically significant in within subject factor (for the main effect: F = 1991; df = 2.34, 252.42; p < .001; for the interaction effect: F = 9.22; df = 4.68, 252.42; p < .001). The test of between subject results shows that the growths between the levels of fishmeal replacement are

also significantly differed from each other (F = 15.02; df = 2, 108; p < .001). Also, the post-hoc test result shows that all the levels are significantly different from each other (p < .05).

Table 285.1 Repeated ANOVA for levels of fishmeal – Tests of within-subjects effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Greenhouse-Geisser	2.566	2.337	1.098	1990.991	.000
Time * LEVEL	Greenhouse-Geisser	.024	4.674	.005	9.220	.000
Error(Time)	Greenhouse-Geisser	.139	252.423	.001		
a. Computed using alpha = .05						

Table 295.2 Repeated ANOVA for levels of fishmeal – Tests of between-subjects effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	9.001	1	9.001	7606.182	.000
LEVEL	.036	2	.018	15.024	.000
Error	.128	108	.001		
a. Computed using alpha = .05					

Table 305.3 Post-hoc Test – Pairwise Comparison between the levels of fishmeal replacement

(I) LEVEL	(J) LEVEL	Mean Difference (I-J)	Std. Error	Sig.[b]
60%	80%	.009*	.003	.010
	100%	.017*	.003	.000
80%	60%	-.009*	.003	.010
	100%	.008*	.003	.039
Based on estimated marginal means				
*. The mean difference is significant at the .05 level.				
b. Adjustment for multiple comparisons: Bonferroni.				

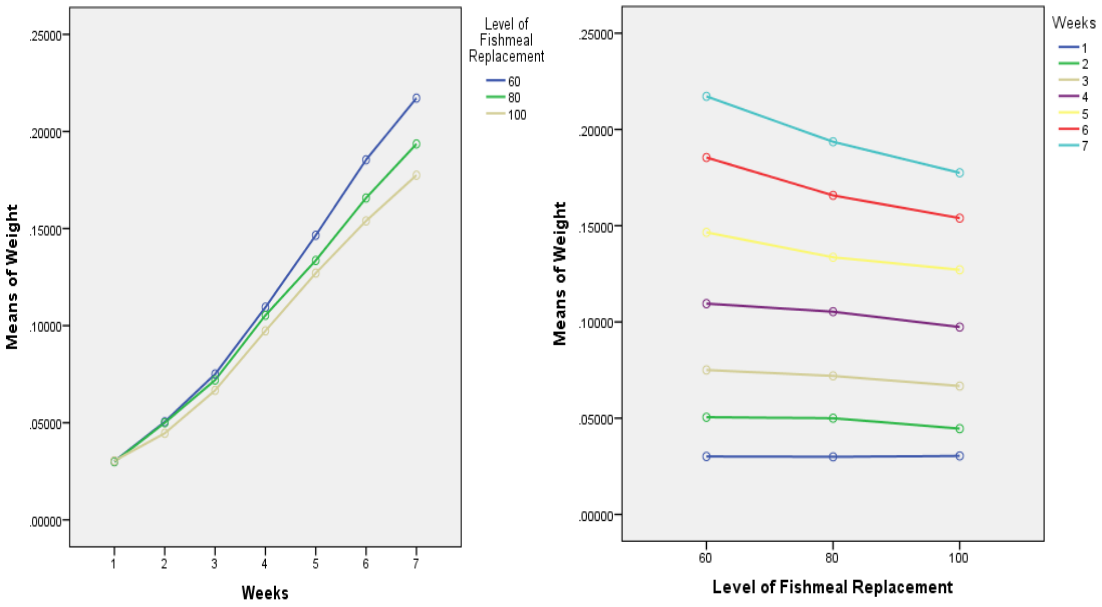

Figure 13. Plot of weight by levels of fishmeal replacement over time

The mean weights for the levels of fishmeal replacement are plotted in Figure 13. The means of weight represents the average weights of diets for each level. As shown in Figure 13, the growth rate was higher when the replacement percentage is 60 and slower when the replacement percentage was 100.

4.2.2.3 Results of the fourth ANOVA model

In this model the within subject factor was time and the between subject factor was the status of lipid (presence or absence). The test results indicate that the main effect of time (in weeks) was found to be statistically significant (F = 1746.51; df = 2.17,

236.51; $p < .001$) in within subject factor, however, the interaction effect between time and lipid was found not be statistically significant ($F = 2.43$; $df = 2.17, 236.51$; $p > .05$). And, the test of between subject results shows that the growths of species between the presence and absence of lipid are not significantly differed from each other ($F = .28$; $df = 1, 109$; $p > .05$). No post-hoc test was observed for this model as the interaction effect was not statistically significant.

Table 316.1 Repeated ANOVA for Lipid – Tests of within-subjects effects

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Greenhouse-Geisser	2.554	2.170	1.177	1746.509	.000
Time * LIPID	Greenhouse-Geisser	.004	2.170	.002	2.431	.086
Error(Time)	Greenhouse-Geisser	.159	236.514	.001		
a. Computed using alpha = .05						

Table 326.2 Repeated ANOVA for Lipid – Tests of between-subjects effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	8.972	1	8.972	6002.166	.000
LIPID	.000	1	.000	.283	.596
Error	.163	109	.001		
a. Computed using alpha = .05					

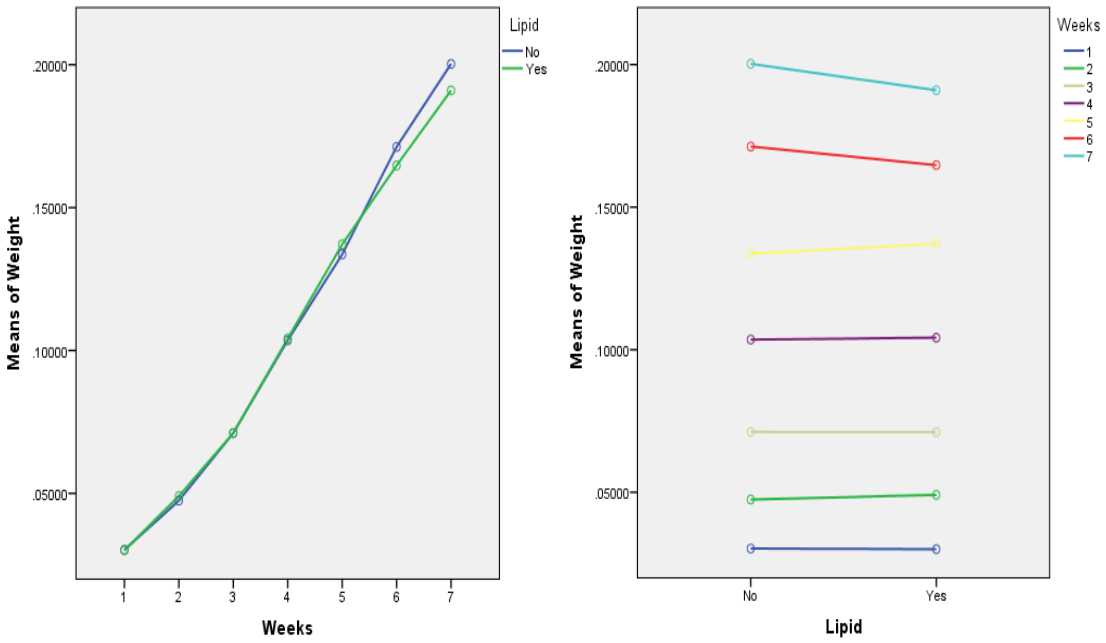

Figure 14. Plot of weight for presence and absence of lipids over time

The mean weights for the presence and absence of lipid are plotted in Figure 14. The means of weight represents the average weights of diets. As shown in Figure 14, comparatively the growth pattern in the absence of lipid was higher than the growth in the presence of lipid, however, this difference was not observed as statistically significant.

Based on the ANOVA models- two to four, the time by species and time by lipid interactions barely fail to attain statistical significance at $p < .05$ alpha level. However, the two-way interaction of time by level is statistically significant ($p < .001$).

Overall, the cumulative growth pattern of the findings of the first ANOVA model in the analysis stage-1 indicate that growth rates for the individual species were significantly lower than growth rates for the controls (see figure 11), although it is interesting to note that the basal diet performed well relative to the individual species regardless the level of fishmeal replacement as well as the presence of lipid. The

analysis of differences between experimental species, levels of fishmeal replacement, and the absence or presence of lipids (overall findings from the second to fourth ANOVA models in the stage-2) indicated that significantly higher growth rates were found for the conditions in which fishmeal replacement level equaled 60% rather than 80% and then 100% respectively.

5. DISCUSSION

The results of this study suggest that the partial replacement of 60% of the fish meal in shrimp diets with three different algal strains of marine proteins can be achieved with minimal reduction in instantaneous shrimp growth; however, findings indicate that shrimp growth rates using the individual algal species were significantly lower than growth rates for the CONTROL or BASAL diets. It is also interesting to note that the basal diet performed well relative to the individual species. The good performance of the BASAL diet with 0% algal replacement supports the implication that the ingredients used to formulate the experimental diets met the nutritional requirements. The analysis of differences between species, levels, and lipids indicated that significantly higher growth rates were found for the conditions in which replacement percentage equaled 60 rather than 100.

The ANOVA indicated that the species of algae did not have a significant effect on performance. Wilson and Poe (1985) state that the dietary protein with the maximum physiological advantage occurs when the amino acid profile of the diet resembles, as closely as possible, that of the consumer. This supports the notion that cultured aquatic organisms are preferably fed with animal proteins; however, this study clearly supports the suitability of using microalgae to meet the nutritional needs of *L. vannamei*. Also, since satisfactory growth was observed for all of the 60 and 80% diets, it can be assumed that other nutritional factors, such as minerals and vitamins, were consistent with apparent good nutritional quality and applicability of the algae.

Weaker growth was exhibited by shrimp fed diets that had 100% fish meal replaced. The cause could be attributed to the amount, or the quality, or both, of the protein offered here, were less than totally adequate. Previous studies determined an optimum dietary protein content of between 28% to 32% for juvenile *L. vannamei* (Kureshy and Davis, 2002). Thus, apparently the protein content of the 100% replacement feeds was not too low for growth but rather, the quality of the protein was likely inadequate for optimum growth. However, the growth could also be

influenced by the clear water laboratory setup for this study. Hopkins *et al*. (1995) found that *L. vannamei* fed diets of 20% and 40% protein had similar growth rates when grown in an intensive outdoor shrimp pond (80 shrimp/meter^2).

The results of this study agree with Davis and Arnold (2000) and Samocha *et al*. (2004) who evaluated diets containing a variety of non-fishmeal protein sources as a replacement diet for *L. vannamei*. They found that replacing fishmeal with co-extruded poultry by-product meal did not adversely affect growth and survival. Davis and Arnold (2004) further refined the diet by replacing menhaden fish oil with plant oils and algae meal. The poor growth, when higher levels of fish meal were replaced, could be caused by a number of reasons. Although the culture environment was adequate in reducing the buildup of harmful toxins, the removal of water each day eliminated the possibility of grazing on phytoplankton or detritus which the shrimp would benefit from and which might support growth. In growout conditions, shrimp can benefit from natural productivity for a good portion of their daily food intake (Weigel, 1994; Kabir Chowdhury *et al*., 2008; Carvajal-Valdes *et al.,* 2012). As previously mentioned, instantaneous growth rates can be improved with frequent feeding which may nullify the poor performance of the high replacement percentage feeds (Carvajal-Valdes *et al.,* 2012). An additional reason could be that the percent protein at the high replacement failed to meet the minimum nutritional requirements, most importantly not just protein, but also amino acids could have been lacking as well.

5.1 Water Quality

The study species, *L. vannamei*, can tolerate salinities of 0.5 to 45 ppt (Bray e*t al.*, 1994; Laramore *et al.*, 2001; Lin and Chen, 2001). During the study, the salinity was kept at near 32 ppt.

Even though a static water system was used, as 90% of the water was replaced daily and 100% of the water was replaced weekly, water conditions were never considered

stressful. Total ammonia-nitrogen (un-ionized plus ionized ammonia as nitrogen) never went above estimated "safety level" for rearing *L. vannamei* juveniles of 3.95 mg/l for ammonia-N and 0.16 mg/l for NH_3-N (un-ionized ammonia as nitrogen) in 35 ppt water (Lin and Chen, 2001). It can be assumed that the test organisms were not adversely affected.

5.2 Survival

Survival of juvenile *L. vannamei* is highest when the animals are kept at temperatures between 20°C and 30°C and salinities above 20 ppt (Ponce-Palafox *et al*., 1997; Lin and Chen, 2001). During this study, the water temperature was kept 26°C to 27°C at and the salinities remained above 30 ppt. As presented earlier in Table 19, all treatments concluded the trial with 71% survival or higher. Variability in juvenile shrimp could affect survival but most losses during this study were attributed to the shrimp being caught above the waterline on the interior of the cup or jumping out of the cup entirely even when lightly fitting covers were used. The survival percentage in this study is similar to some studies utilizing methods consistent with this study although most studies include twenty or more larvae in larger containers (Castille *et al*., 1993; Nuñez *et al*., 2002). However, higher survival rates (>90%) are often found in growth trials that are held in outdoor tanks (Davis *et al*., 2004; Cruz-Suárez *et al*., 2007) or ponds (Balakrishnan *et al*., 2011).

5.3 Nutrition

Efficient growth in animals requires that essential nutrients are in forms that are biologically utilizable and in the appropriate amounts (Ammerman *et al*., 1995). As previously mentioned, research over the past 20 years has provided a good understanding of primary nutrient requirements (Davis *et al*., 2004). Any replacement of ingredients, including supplementing plant protein for marine protein, should be part of a replacement strategy that considers nutritional requirements for protein, essential amino acids, fatty acids, minerals and vitamins (Penaflorida, 1989; Mente *et al*., 2002; Davis *et al*., 2004). A challenge for nutrition studies is to apply

small scale research to large grow-out facilities. This study focused on protein as a key factor in the survival and growth of *L. vannamei*, as highlighted in the following section.

5.3.1 Protein

Protein is a major limiting nutrient for growth (Kureshy and Davis, 2000). The composition of the natural biofloc and the offered feed must meet a number of nutritional requirements that are critical for the survival, growth, and reproductive capacity and a great deal of research has been dedicated in determining those requirements (Andrews *et al.*, 1972; Jauncey, 1982; Hopkins *et al*., 1994; McIntosh *et al.*, 2000; McIntosh *et al.*, 2001; Tacon *et al*., 2002; Patnaik *et al.*, 2006). Protein can be the determining factor in the growth and survival *L. vannamei*. For juvenile *L. vannamei*, Colvin and Brand (1977) reported less than 30% to be the protein requirement while Kureshy and Davis (2000) found a maximum protein requirement at 32% for juveniles and sub-adults.

The results of this study support the previous research that optimal dietary protein levels may be in the range of 25-34% by weight. Diets in this study with less than 25% displayed slower growth but survival was still high (Table 19). Reduced growth will increase the feed conversion rate (Colvin and Brand, 1977) which will impact the profitability and success of aquaculture ventures. Studies have shown that diets containing lower percent protein can perform as well or better than diets containing higher percent protein if the animals are fed to satiation (Davis, 2005). A 30% protein diet fed to satiation can perform as well as a 40% protein diet fed only to 75% of satiation (Davis, 2005). Additionally, aquaculture facilities have been able to utilize lower protein feeds (25%) if sufficient natural production and biofloc is available (Hopkins *et al*., 1995; Tacon and Barg, 1998; Martinez-Cordova *et al*., 2002). Martinez-Cordova *et al*. (2002) found no significant differences between feed conversion ratio, total growth, or survival when a low-protein (25%) and a high-protein diet were used during a 16-week study carried out in outdoor earthen ponds.

In general, it would be expected that diets with higher percent protein would outperform those with lower but that did not occur during this study. The analysis of differences between species, levels, and the absence or presence of lipids indicated that significantly higher growth rates were found for the conditions in which the level equaled 60 rather than 100; however, lipids and species did not have significant effects on performance (Figures 20, 21).

5.4 Replacement Feed Ingredients

Sources of fishmeal have traditionally included fishmeal, squid, crab, and bivalves (Guillaume *et al*., 1999). Replacement diets have been investigated in efforts to minimize the dependence on marine proteins (Lawrence and Castille, 1993; Samocha *et al.*, 2004; Davis and Arnold, 2004).

Consequently, as the cost of fish meal increased in the past few years, more research into finding a replacement or a partial substitute for the fish meal from diets has occurred (Davis and Arnold, 2000; Samocha *et al.*, 2004). Studies using soybean meal as a replacement or partial substitute have resulted in both success and failure (Lim and Dominy, 1990; Samocha *et al.*, 2004) and more studies are ongoing. Generally it is accepted now that soybean meal can replace a large amount of fish meal without a loss in growth or survival rates (Samocha *et al.*, 2004). Lim and Dominy (1990; 1992) found that soybean meal could effectively replace up to 42% of fish meal for *L. vannamei*.

Research has been conducted to determine if microalgae would be a suitable as a partial or complete replacement in *L. vannamei* feeds (Hanel *et al*., 2007). In the study conducted by Hanel *et al.* (2007), fish meal was replaced with *Spirulina platensis* as it contains 60-70% protein dry weight making it attractive to use. The overall results of the study were positive as the growth of animals fed diets with fish meal replaced were not statistically significant than the commercial diet.

In this study, 60% replacement did perform significantly better, for growth, than 80 or 100%. However, the control and basal diet performed significantly better than any of the experimental diets with algae.

5.5 Growth Rates

Growth rates of the experimental shrimp observed during the present trials ranged from 9.24 to 17.30% per day instantaneous growth rates. The similarly high growth rates obtained with meals replaced with 60% and commercial shrimp diet (CONTROL) and basal diet shows that a replacement of a large amount of fishmeal can be achieved in an aquatic animal diet. Regarding the weak growth rates of the diets at the higher percentage levels (80 and 100% replacement), it can be assumed that the quality and or quantity of the protein offered here was not optimum for juvenile *Litopenaeus vannamei*. The ability for a shrimp to forage additional floc from the benthos may alter final growth numbers and is an essential component of a growout facility's farming system and feeding strategy (Tacon *et al*., 2004).

For the larval forms of many species, highly variable rates of growth are found between larvae raised under identical laboratory conditions (Pace *et al*., 2005). The complex nature of the processes that are regulated by both endogenous biological and exogenous factors regulate the growth of larvae of invertebrates (Moran and Manahan, 2004; Pace *et al*., 2005). The number of replicates used in the present trial are designed to reduce the impact of those variations; however, the endogenous biological factors cannot be determined.

5.6 Variability of Growth between Individual Shrimp

As with many species, there is variability between different broodstock populations which has resulted in a great deal of research on hatchery science (Chamberlain and Pettibone, 1990; Castille *et al*., 1993). To minimize this factor, the shrimp used in

this study were all taken from broodstock following multiple generations of breeding and selection for growth. However, as with any natural population, outliers may exist. This study evaluated the survival and growth of individuals and their response to varying feeds. It does not appear that the stock used for this study varied genetically which would influence their growth.

5.7 Correlations between Growth and Algal Species

The results of the analysis indicated that the fastest growth rate for all species was obtained with a 60% replacement diet. The experimental diets with substitutions above 80% resulted in diminished performance; however, final survival was acceptable for all treatments.

The results of this study are consistent to earlier research which found that the level of protein in a feed is the major driver in growth. Patnaik *et al*. (2006) replaced fish meal with a combination of co-extruded soybean and poultry by-product and different levels of heterotrophic algae, *Schizochytrium* sp. and *Mortierella alpina*. After the 15-week study of *L. vannamei* (0.66 ± 0.06 g), growth and survival values were not significantly affected by the replacement diet and performed as well as the control diet.

5.8 Correlations between Growth and Lipid Content

Comparisons between diets considered the effects of species, replacement %, and lipids within an ANOVA framework. The ANOVA considers the effects of:

a.) species, pooled across Lipid/Non-Lipid and Replacement %
b.) Lipid versus non-Lipid, pooled across species and percent replacement diet
c.) % replacement diet, pooled across species and Lipid vs non-Lipid

The results of the analysis indicated that the absence or presence of lipids did not have significant effects on performance. As research has established the importance of lipids in diets, it needs to be addressed as to why lipid content did not influence the

growth rates of shrimp significantly in this study. Dietary lipids are a source for essential fatty acids, phospholipids, sterols and carotenoids and all contribute to health metabolic function (Lim *et al*., 1997; González-Félix and Perez-Velazquez, 2002).

5.9 Energy Demand and the Future of Aquaculture

Environmental impacts and depletion of fossil fuels has generated resurgence into research towards alternative and renewable sources of energy (Torres *et al*., 2013; Collet *et al*., 2014). The pressure to find new sources of energy grows greater each year as the population of the world increases and the standard of living in developing countries gets better. It is clear that with sustainable energy policy, a sustainable food program must be developed to ensure proper nutrition is available for people worldwide. Almost every year since the 1980s, global aquaculture production expanded at an average rate of more than 8 percent, from 5.2 million tons in 1981 to 62.7 million tons in 2011 (FAO, 2013). The industry has learned from earlier mistakes in regards to biosecurity and has supported the recovery of many global marine fisheries (Lightner, 2005; 2011; Turkmen and Toksen, 2007). An opportunity exists to further evolve the global aquaculuture industry as we look to reduce the pressure on fish meal for feed. Although many terrestrial protein substitutes are suitable to different degrees (Samocha, 2004), the development of third-generation biofuels from algae can help solve a portion of the energy challenge and support an increase of aquaculture activities as it has the potential to decrease feed costs as a portion of fish meal in feed is replaced by the algae biomass after lipid removal.

6. CONCLUSIONS

Four conclusions can be reached from the research conducted:

- The apparent protein and energy requirements for growth in *L. vannamei* were met by all treatments with the three species of algae (*Chaetoceros calcitrans*, *Nannochloropsis salina*, and *Pavlova sp*).
- Replacement of fish protein in the diets at the 60% level provided a diet that performed better in *L. vannamei* growth than replacement at the 80 or 100% level.
- Control and basal diets provided statistically higher growth than any of the experimental diets.
- Plant proteins may have a place in removing fish meal from *L. vannamei* diets but complete replacement may not be suitable.

This study indicates that acceptable growth and survival can be achieved by partial substitution of fish meal in diets for juvenile *Litopenaeus vannamei*. The favorable response of shrimp to algal meals in the present experiment is likely due to the fact that all of the ingredients used have been reviewed for digestibility as well as a lack of apparent palatability problems. Each of the algal strains used has been used previously for larvae culture and the favorable response seen in this study may not occur if a strain is used that leads the larvae to have digestibility problems or refuse to consume the diet due to poor flavor or palatability.

In the present study, growth and survival were either improved or were not substantially influenced by the replacement of fish meal with algal biomass at the 60% level. As the biofuel industry matures, the cost of algal meal will continue to decrease due to the additional supply and should be competitively priced versus fish meal; however, the cost-effectiveness of substituting it for fish meal will vary depending on the location and local costs of the ingredients. A level at or below the tested level could provide similar positive growth and should be considered in future research.

While these preliminary results suggest that fish proteins can be replaced to a large degree by plant proteins, more research needs to be undertaken before the results can be used to supplement *in situ* trials involving *L. vannamei*. The dietary requirements of juvenile *L. vannamei* are different in adulthood and further growout studies may indicate the need to adjust the feed formula to acquire maximum growth and feed efficiency. The optimal level of neutral (e.g. TAG) and polar (PL) lipids in shrimp diets and their digestibility in adult shrimp should be investigated. The metabolic pathways of shrimp at growout facilities differ from the conditions in a laboratory and often produce different results (Castille *et al*., 1993; Tacon, 1996). Further investigation using byproducts from algal feedstock can benefit the aquaculture industry and the economy as a whole.

The success of shrimp aquaculture depends greatly on its ability to develop feeds that can meet certain growth requirements, its sustainability, and its cost effectiveness. The development of better farming practices over the years, including the use of probiotics in feed, has decreased the impact of farms on the environment and reduced the incidence of disease (Wyaban, 2009; Lakshmi *et al*., 2013). As feed is a key component in the overall pond management strategy, it has taken longer to develop feeds that meet the above listed criteria. This study provides additional information on the inclusion of algae into shrimp feeds that may be beneficial to the shrimp aquaculture industry.

Furthermore, there is a need for carbon sequestration from coal fire power plants here in the US and abroad. As algae consume carbon dioxide during autotrophic cell growth to regenerate biomass and to reproduce, the injection of flue gases from power plants can increase the biomass productivity by 30% as compared to the injection of pure carbon dioxide (Sayre, 2010). Excess carbon that is not used for biomass is stored as neutral lipids which can be harvested and converted into biodiesel. The integration of algal ponds with power plants has been already established but the utilization of the algal biomass after lipid extraction for aquaculture diets can further

support the initiative. The implementation of this system has greater merit in developing countries that heavily rely on subsistence farming for primary protein sources.

In the end, the feasibility to leverage the production of microalgae-based biodiesel and aquaculture does depend on the economics. The true belief, or to a lesser degree – the optics, of replacing fish proteins with algae paste may convince some aquaculturists to adopt this strategy but the adoption of the use of algae paste must make economic sense. The final price that is paid at the store may be higher than traditionally-raised aquaculture products as this may be perceived as a "greener" option and people are willing to pay more for those products labeled as organic or welfare-raised (Budak *et al*., 2006; Olesen *et al*., 2010).

Future Research

Further work may provide information on how byproducts from biofuel production can be used by feed manufacturers. The results of this study suggest that the maximum replacement is less than 60% as compared to the growth exhibited during the study by the shrimp on the control and basal diets. Additionally, further studies evaluating the composition of algal biomass after using a high efficiency lipid removal process is recommended as it is evident that the method utilized during this study is not commercially viable due to the time to process and the incomplete lipid removal.

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Appendix A. Algae Species
National Center for Marine Algae and Microbiota
http://ncma.bigelow.org

CCMP459 *Pavlova cf sp.*
Class: Prymnesiophyceae

Collected:	07/11/1980	Isolated by:	Provasoli, L
Collection Site:	38.7020N 72.3667W Oceanus cruise 83 station II	Isolated date:	Not Available
Ocean:	North Atlantic	Identified by:	Not Available
Sea:	Gulf Stream	Deposited by:	Provasoli, L
Nearest Continent:	North America	Deposited date:	02/24/1983
Other Information:	20-21˚C, 20-25m on nylon rope,	Initial Axenic date:	Not Available
		Initial Axenic by:	Not Available
		Currently Axenic:	Yes

Culture medium:	L1, f/2-Si, ASM4, Prov	Species synonyms:	IIF1, IIF1AX
Temp. range at CCMP:	22-26°C	Name synonyms:	none
Cell length:	4 - 8 µm	Authentic/Type species:	No
Cell width:	4 - 5 µm		
Bioluminescent:	No		

Appendix A. Algae Species (Continued)

CCMP369 *Nannochloropsis salina* (Hibberd)

Class Eustigmatophyceae

Collected:	Lewin,R 06/22/1986	Isolated by:	Lewin, R
Collection Site:	41.6000N 71.4000W Narragansett Bay, Rhode Island, USA (approx.)	Isolated date:	1986
Ocean:	North Atlantic	Identified by:	Andersen, RA
Sea:	Narragansett Bay	Deposited by:	Lewin, R
Nearest Continent:	North America	Deposited date:	04/10/1990
Other Information:	pool above HWM.turbid water, probably component of picopleuston	Initial Axenic date:	1986
		Initial Axenic by:	Lewin, RA
		Currently Axenic:	Yes

Culture medium:	f/2-Si, f/2 agar, L1 - Si	Species synonyms:	278-02
Temp. range at CCMP:	22-26°C	Name synonyms:	none
Cell length:	3 - 8 µm	Authentic/Type species:	No
Cell width:	2 - 4 µm		
Bioluminescent:	No		

Appendix A. Algae Species (Continued)

CCMP1315 *Chaetoceros calcitrans* (Paulsen)

Class Coscinodiscophyceae

Collected:	Umebayashi, O 1960	Isolated by:	Umebayashi, O
Collection Site:	collection site unknown	Isolated date:	1960
Ocean:	Unknown	Identified by:	Not Available
Sea:	Unknown	Deposited by:	Booth, B
Nearest Continent:	Unknown	Deposited date:	02/17/1983
Other Information:	from a culture of Porphyra	Initial Axenic date:	Not Available
		Initial Axenic by:	Not Available
		Currently Axenic:	Yes
Culture medium:	f/2, L1	Species synonyms:	CCAL, NEPCC590, CCAP 1010/11
Temp. range at CCMP:	22-26°C	Name synonyms:	none
Cell length:	3 - 7 µm	Authentic/Type species:	No
Cell width:	3 - 5 µm		
Bioluminescent:	No		

Appendix B. F/2 Algae Growth Medium: Composition of Mineral Premix

Nutrient Name	Unit of Measure	Value
Calcium	%	0.08
Phosphorus	%	1.08
Sodium	%	38.90
Potassium	%	1.20
Magnesium	%	0.56
Iron	PPM	72
Zinc	PPM	46072
Manganese	PPM	1100
Copper	PPM	12024
Arginine	%	0.56
Histidine	%	0.24
Isoleucine	%	0.44
Leucine	%	0.96
Lysine	%	0.41
Methionine	%	0.16
Methionine/Cysteine	%	0.32
Phenylalanine	%	0.40
Phenyl-Tyrosine	%	0.80
Threonine	%	0.36
Tryptophan	%	0.12
Valine	%	0.56
Retinol	IU/KG	600000
Cholecalciferol	IU/KG	500000
Tocopherol	MG/KG	40012
Thiamine	MG/KG	7056
Riboflavin	MG/KG	11001
Pyridoxine	MG/KG	22003
Niacin	MG/KG	22096
Pantothenic Acid	MG/KG	8208
Biotin	MG/KG	200
Folic Acid	MG/KG	5000
Cyanocobalamin	MG/KG	40

% = Percent
PPM = Parts Per Million
IU/KG = International Units Per Kilogram
MG/KG = Milligrams Per Kilogram

Appendix C. F/2 Algae Growth Medium: Composition of Vitamin Premix

Nutrient Name	Unit of Measure	Value
Calcium	%	0.08
Phosphorus	%	1.08
Sodium	%	38.90
Potassium	%	1.20
Magnesium	%	0.56
Iron	PPM	72
Zinc	PPM	72
Manganese	PPM	5300
Copper	PPM	24
Arginine	%	0.56
Histidine	%	0.24
Isoleucine	%	0.44
Leucine	%	0.96
Lysine	%	0.41
Methionine	%	0.16
Methionine/Cysteine	%	0.32
Phenylalanine	%	0.40
Phenyl-Tyrosine	%	0.80
Threonine	%	0.36
Tryptophan	%	0.12
Valine	%	0.56
Retinol	IU/KG	1100000
Cholecalciferol	IU/KG	500000
Tocopherol	MG/KG	40012
Thiamine	MG/KG	3556
Riboflavin	MG/KG	5551
Pyridoxine	MG/KG	11006
Niacin	MG/KG	11096
Pantothenic Acid	MG/KG	4104
Biotin	MG/KG	100
Folic Acid	MG/KG	2500
Cyanocobalamin	MG/KG	10

% = Percent
PPM = Parts Per Million
IU/KG = International Units Per Kilogram
MG/KG = Milligrams Per Kilogram

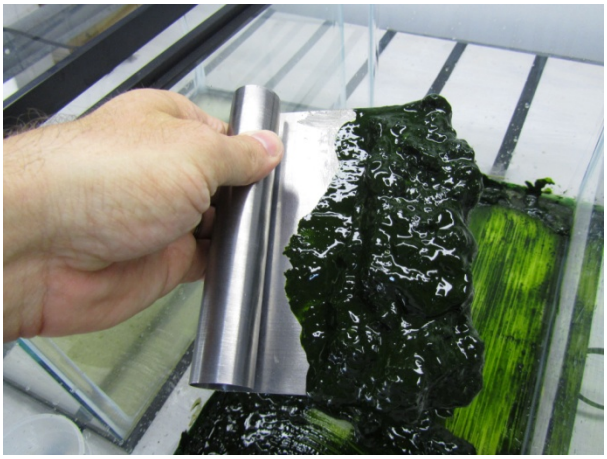
Appendix D. F/2 Medium: Kent F/2 Algal Food, Parts A & B

"A" Guaranteed Analysis	**Percentage (%)**
Iron (Fe)	1.3
Manganese (Mn)	0.034
Cobalt (Co)	0.002
Zinc (Zn)	0.0037
Molybdate (Mo)	0.0009
Copper (Cu)	0.0017
Ingredients: Ferric Chloride, EDTA, Cobalt Chloride, Copper Sulfate, Sodium Sulfate, Sodium Molybdate	

"B" Guaranteed Analysis	**Percentage (%)**
Nitrogen (N)	15.0
Phosphate (P_2O_5)	2.0
Vitamin B_1	0.07
Vitamin B_{12}	0.0002
Biotin	0.002
Copper (Cu)	0.0017
Ingredients: Sodium Nitrate, Monosodium Phosphate, Thiamine Hydrochloride (Vitamin B_1), Vitamin B_{12}, Biotin	

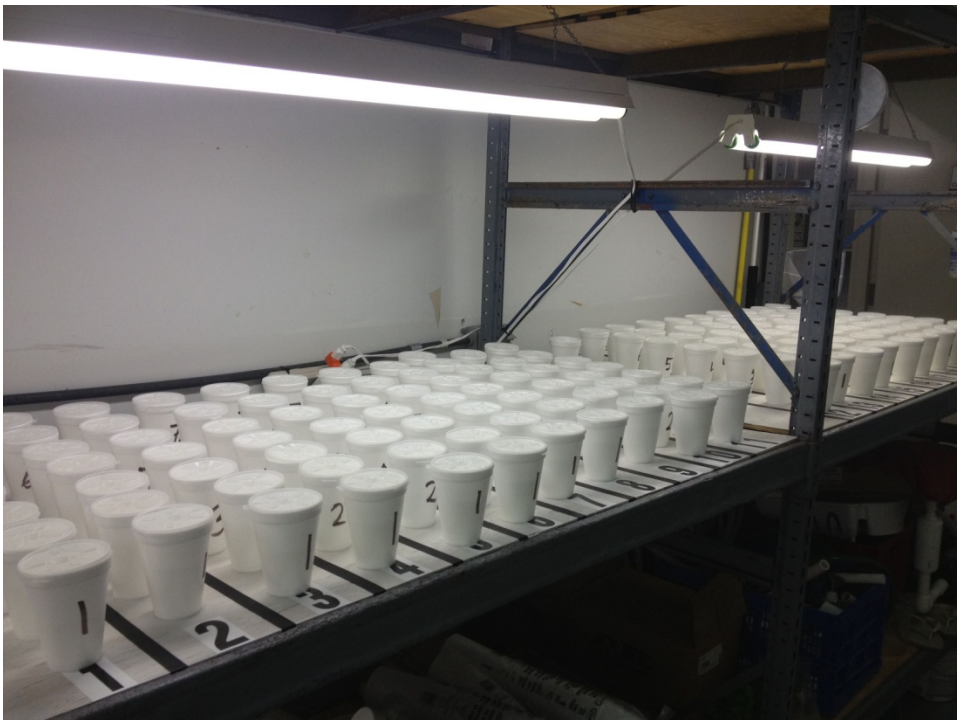
Appendix E. Flow Chart of Algae Culture to Diet Constituent

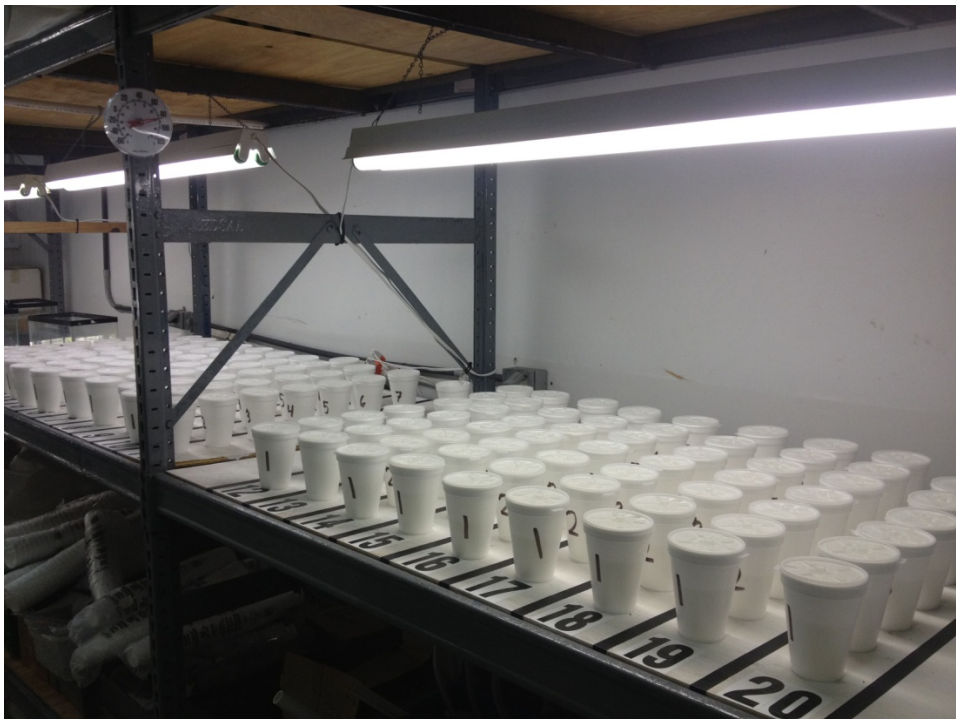
From culture to drying station



Appendix F. Feed pellets after extrusion
Example of pellets prior to breaking down to crumble

Appendix G. Experimental Setup**:** Each diet was randomly assigned to one of twenty rows for the feed trial. Each diet had seven replicates





Appendix H. Weight of *L. vannamei* fed a diet of 60% *Chaetoceros calcitrans* (Chaeto60)

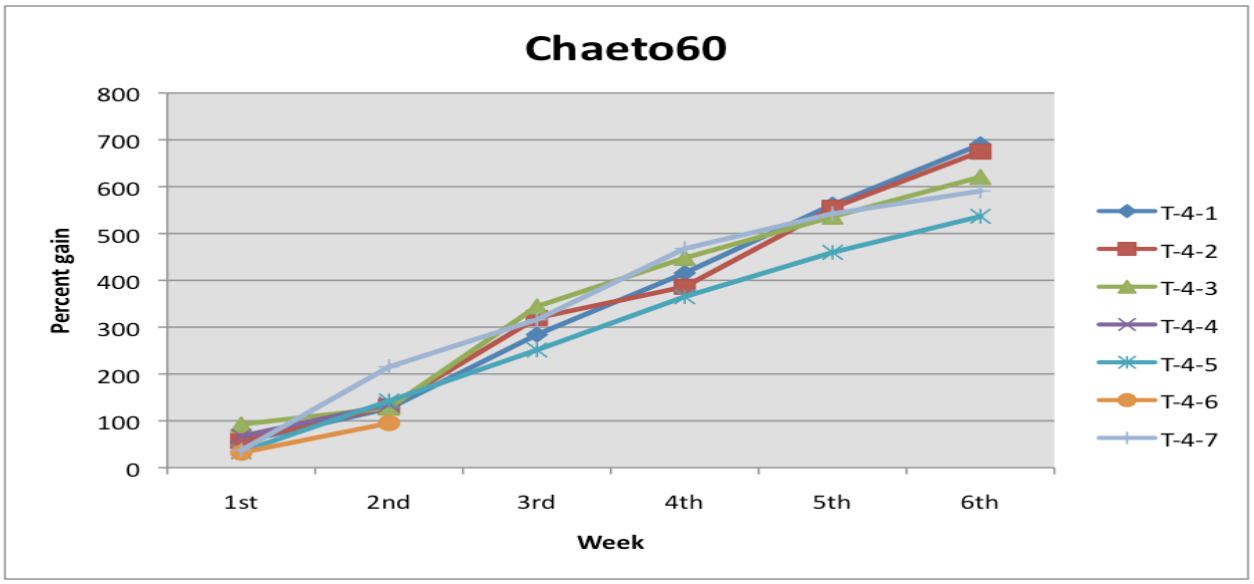
Algal Species: *Chaetoceros calcitrans* **Grid Position**: 4
Treatment: Chaeto60 **Treatment #**: 1

T-4. Chaeto60 – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-4-1	0.0283	0.0438	0.0641	0.1087	0.1459	0.1872	0.2236
T-4-2	0.0314	0.0493	0.0724	0.1318	0.1527	0.2056	0.2433
T-4-3	0.0276	0.0531	0.0629	0.1226	0.1512	0.1755	0.1989
T-4-4	0.0312	0.0521	0.0734				
T-4-5	0.0349	0.0467	0.0846	0.1226	0.1623	0.1953	0.2222
T-4-6	0.0348	0.0459	0.0679				
T-4-7	0.0276	0.0378	0.0870	0.1148	0.1567	0.1773	0.1906
Average	0.0308	0.0470	0.0732	0.1201	0.1538	0.1882	0.2157
Std. Dev.	0.0032	0.0052	0.0095	0.0088	0.0061	0.0126	0.0211

T-4. Chaeto60 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-4-1	55	127	284	416	561	690
T-4-2	57	131	320	386	555	675
T-4-3	92	128	344	448	536	621
T-4-4	67	135				
T-4-5	34	142	251	365	460	537
T-4-6	32	95				
T-4-7	37	215	316	468	542	591
Average	53	139	303	416	531	623
Std. Dev.	22	37	36	42	41	63



Appendix I. Weight of *L. vannamei* fed a diet of 60% *Chaetoceros calcitrans* (Chaeto60L)

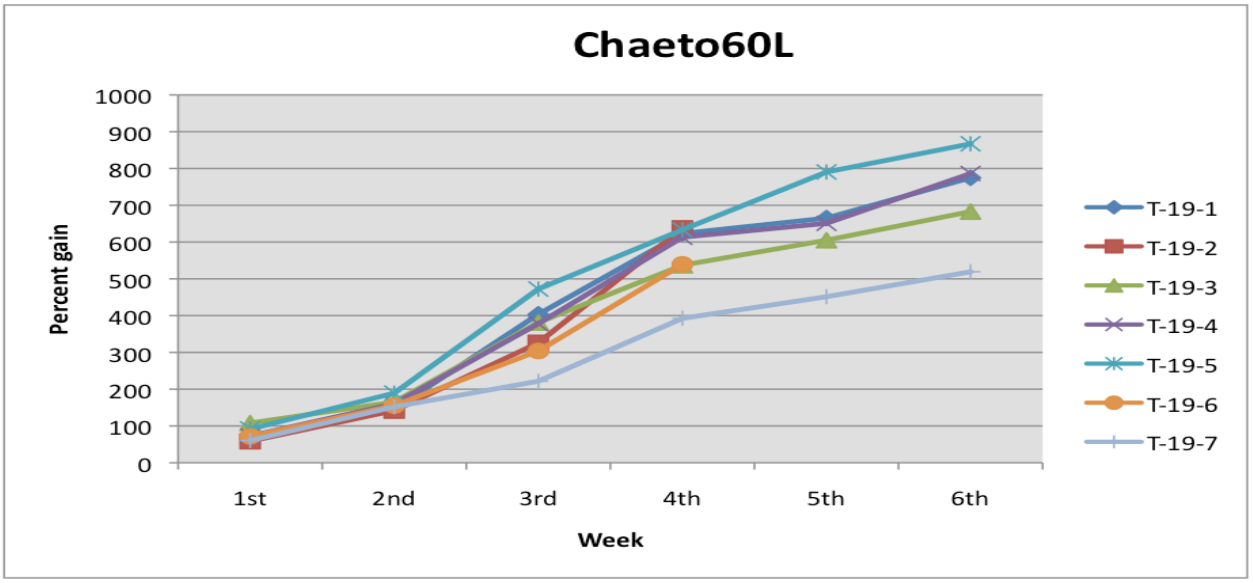
Algal Species: *Chaetoceros calcitrans* **Grid Position**: 19
Treatment: Chaeto60L **Treatment #**: 2

T-19. Chaeto60L – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-19-1	0.0278	0.0459	0.0683	0.1400	0.2011	0.2127	0.2432
T-19-2	0.0294	0.0466	0.0711	0.1252	0.2167		
T-19-3	0.0314	0.0653	0.0833	0.1511	0.2000	0.2214	0.2458
T-19-4	0.0312	0.0539	0.0812	0.1492	0.2225	0.2343	0.2765
T-19-5	0.0271	0.0519	0.0784	0.1551	0.1987	0.2413	0.2621
T-19-6	0.0283	0.0486	0.0723	0.1143	0.1809		
T-19-7	0.0296	0.0475	0.0746	0.0952	0.1458	0.1632	0.1833
Average	0.0293	0.0514	0.0756	0.1329	0.1951	0.2146	0.2422
Std. Dev.	0.0016	0.0068	0.0055	0.0222	0.0256	0.0308	0.0356

T19. Chaeto60L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-19-1	65	146	404	623	665	775
T-19-2	59	142	326	637		
T-19-3	108	165	381	537	605	683
T-19-4	73	160	378	613	651	786
T-19-5	92	189	472	633	790	867
T-19-6	72	155	304	539		
T-19-7	60	152	222	393	451	519
Average	75	159	355	568	633	726
Std. Dev.	18	16	80	88	122	133



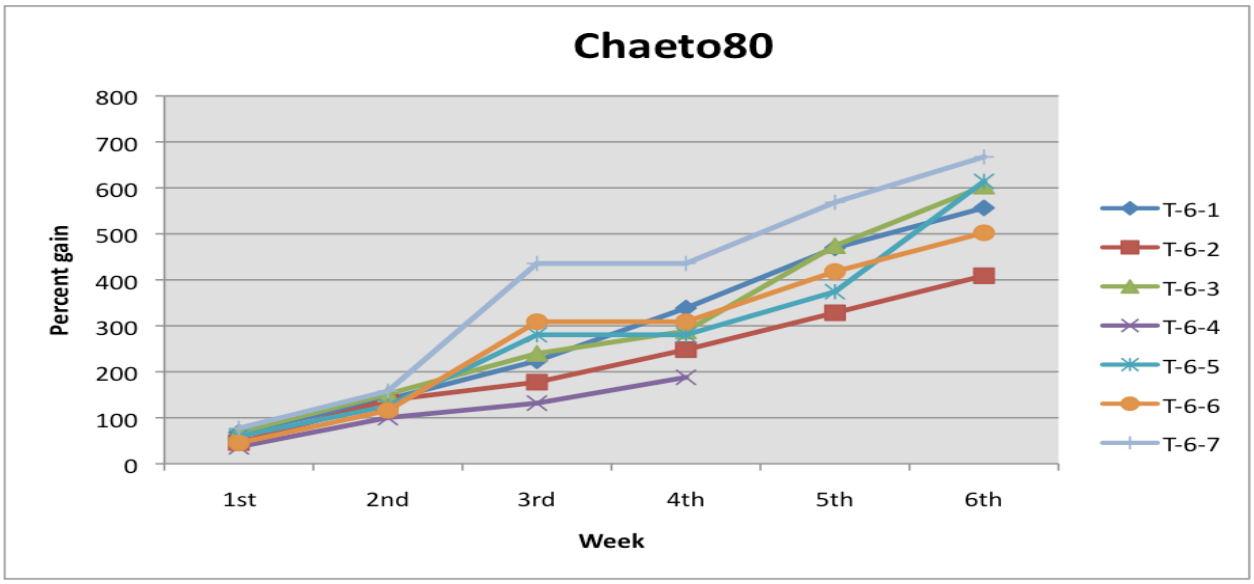
Appendix J. Weight of *L. vannamei* fed a diet of 80% *Chaetoceros calcitrans* (Chaeto80)

Algal Species: *Chaetoceros calcitrans* **Grid Position**: 6
Treatment: Chaeto80 **Treatment #**: 3

Week 2	Week 3	Week 4	We
0.0479	0.0684	0.0923	0.1
0.0428	0.0698	0.0813	0.1
0.0456	0.0682	0.0921	0.1
0.0534	0.0781	0.0904	0.1
0.0476	0.0672	0.1134	0.1
0.0492	0.0732	0.1386	0.1
0.0468	0.0681	0.1414	0.1
0.0476	0.0704	0.1071	0.1
0.0033	0.0039	0.0245	0.0

T-6. Chaeto80 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-6-1	68	140	224	339	469	556
T-6-2	46	138	177	248	328	409
T-6-3	68	152	240	289	474	604
T-6-4	37	100	132	188		
T-6-5	60	126	281	281	374	614
T-6-6	45	116	309	309	418	502
T-6-7	77	158	436	436	569	667
Average	57	133	257	298	439	559
Std. Dev.	15	20	99	77	85	92



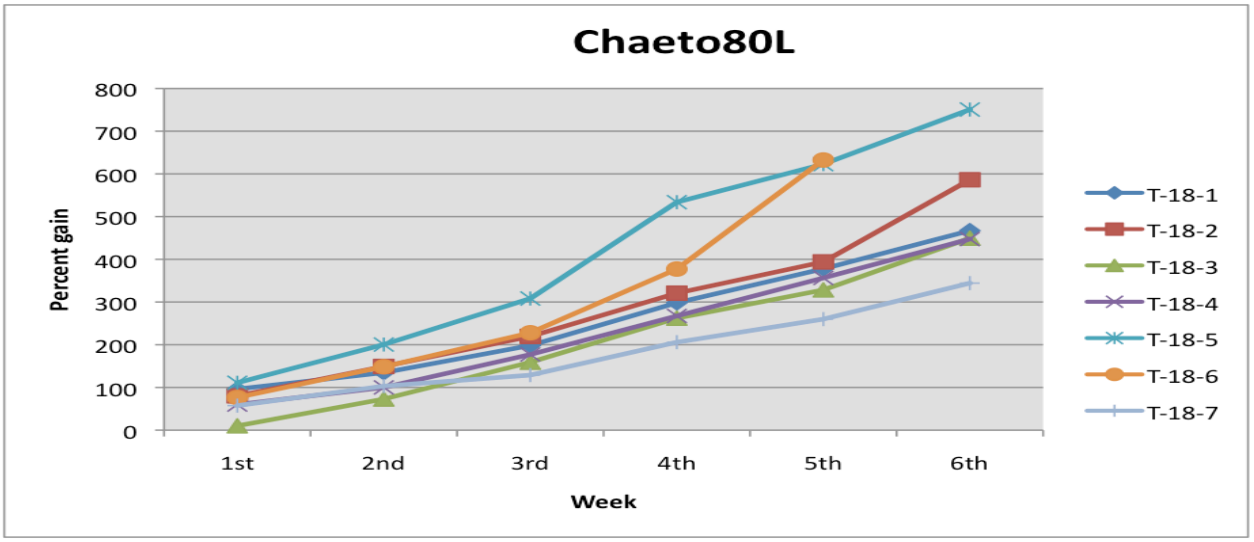
Appendix K. Weight of *L. vannamei* fed a diet of 80% *Chaetoceros calcitrans* (Chaeto80L)

Algal Species: *Chaetoceros calcitrans* **Grid Position**: 18
Treatment: Chaeto80L **Treatment #**: 4

Week 2	Week 3	Week 4	We
0.0679	0.0812	0.1032	0.1
0.0539	0.0745	0.0955	0.1
0.0349	0.0548	0.0821	0.1
0.0539	0.0674	0.0934	0.1
0.0462	0.0658	0.0894	0.1
0.0473	0.0666	0.0879	0.1
0.0452	0.0583	0.0657	0.0
0.0499	0.0669	0.0882	0.1
0.0102	0.0090	0.0119	0.0

T-18. Chaeto80L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-18-1	96	135	198	298	378	467
T-18-2	80	149	219	321	394	586
T-18-3	10	73	159	262	328	450
T-18-4	60	100	177	267	356	447
T-18-5	111	200	308	534	623	751
T-18-6	76	149	228	377	632	
T-18-7	57	103	129	206	260	344
Average	70	130	203	324	425	508
Std. Dev.	33	42	58	107	145	142



Appendix L. Weight of *L. vannamei* fed a diet of 100% *Chaetoceros calcitrans* (Chaeto100)

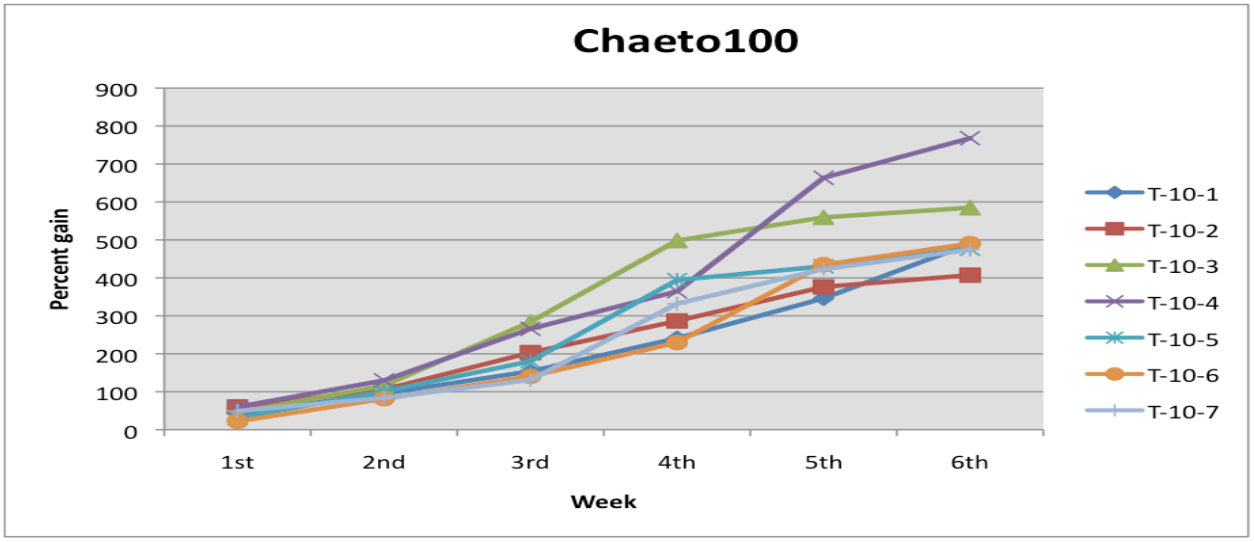
Algal Species: *Chaetoceros calcitrans* **Grid Position**: 10
Treatment: Chaeto100 **Treatment #**: 5

T-10. Chaeto100 – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-10-1	0.0330	0.0473	0.0645	0.0838	0.1125	0.1472	0.1945
T-10-2	0.0320	0.0512	0.0659	0.0970	0.1237	0.1524	0.1623
T-10-3	0.0283	0.0412	0.0612	0.1087	0.1693	0.1866	0.1938
T-10-4	0.0234	0.0375	0.0538	0.0856	0.1087	0.1787	0.2031
T-10-5	0.0318	0.0415	0.0639	0.0892	0.1572	0.1687	0.1834
T-10-6	0.0351	0.0428	0.0638	0.0845	0.1157	0.1877	0.2072
T-10-7	0.0319	0.0476	0.0587	0.0738	0.1378	0.1669	0.1832
Average	0.0308	0.0442	0.0617	0.0889	0.1321	0.1697	0.1896
Std. Dev.	0.0038	0.0047	0.0042	0.0111	0.0235	0.0158	0.0150

T-10. Chaeto100 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-10-1	43	95	154	241	346	489
T-10-2	60	106	203	287	376	407
T-10-3	46	116	284	498	559	585
T-10-4	60	130	266	365	664	768
T-10-5	31	101	181	394	431	477
T-10-6	22	82	141	230	435	490
T-10-7	49	84	131	332	423	474
Average	44	102	194	335	462	527
Std. Dev.	14.2	17.2	60.4	94	111	118



Appendix M. Weight of *L. vannamei* fed a diet of 100% *Chaetoceros calcitrans* (Chaeto100L)

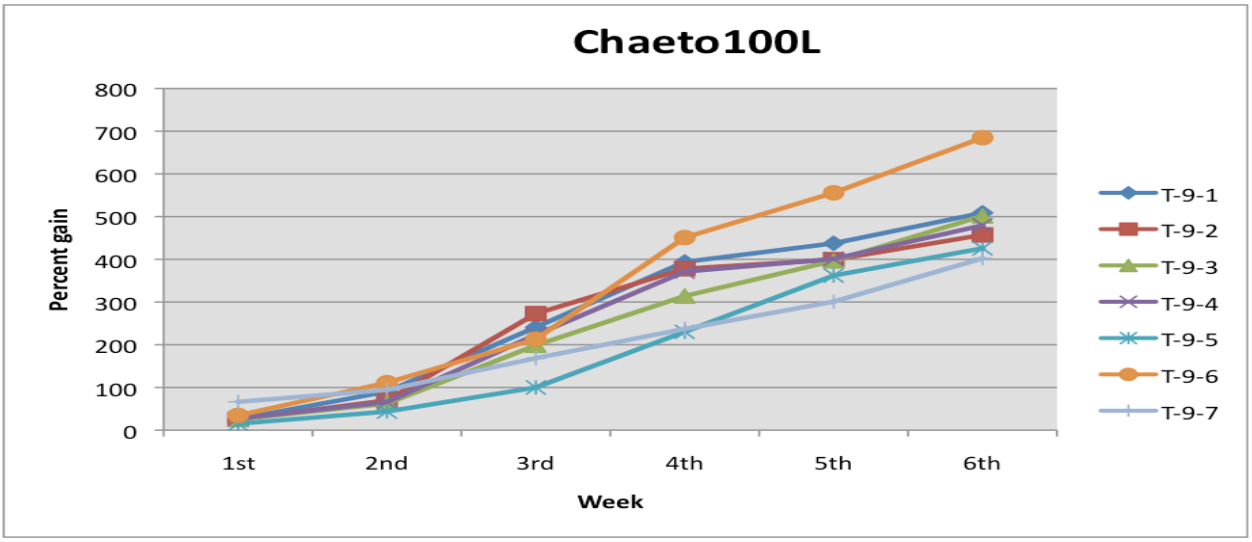
Algal Species: *Chaetoceros calcitrans* **Grid Position**: 9

Treatment: Chaeto100L **Treatment #**: 6

Week 2	Week 3	Week 4	We
0.0347	0.0523	0.0938	0.1
0.0415	0.0562	0.1234	0.1
0.0385	0.0498	0.0917	0.1
0.0375	0.0489	0.0954	0.1
0.0312	0.0389	0.0542	0.0
0.0362	0.0568	0.0842	0.1
0.0524	0.0612	0.0846	0.1
0.0389	0.0520	0.0896	0.1
0.0068	0.0072	0.0204	0.0

T-9. Chaeto100L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-9-1	26	90	241	394	437	509
T-9-2	25	70	273	378	399	457
T-9-3	25	62	199	314	396	502
T-9-4	27	66	223	371	401	480
T-9-5	15	44	100	230	362	426
T-9-6	35	111	213	451	556	685
T-9-7	66	94	169	237	301	402
Average	31	77	203	339	408	494
Std. Dev.	16	23	56	83	78	92



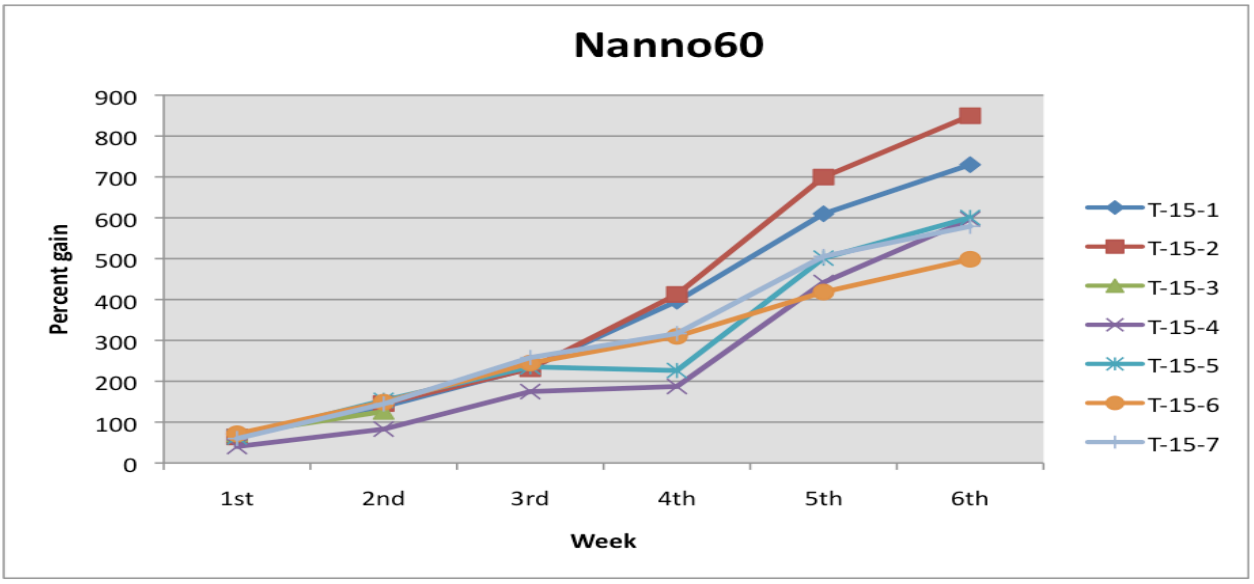
Appendix N. Weight of *L. vannamei* fed a diet of 60% *Nannochloropsis salina* (Nanno60)

Algal Species: *Nannochloropsis salina* **Grid Position**: 15
Treatment: Nanno60 **Treatment #**: 7

Week 2	Week 3	Week 4	We
0.0487	0.0683	0.0954	0.1
0.0462	0.0692	0.0932	0.1
0.0538	0.0710		
0.0484	0.0631	0.0948	0.0
0.0511	0.0790	0.1046	0.1
0.0573	0.0831	0.1151	0.1
0.0438	0.0670	0.0981	0.1
0.0499	0.0715	0.1002	0.1
0.0046	0.0070	0.0083	0.0

T-15. Nanno60 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-15-1	70	138	232	396	610	730
T-15-2	64	145	230	412	700	850
T-15-3	71	126				
T-15-4	40	83	175	187	442	597
T-15-5	64	153	235	226	501	601
T-15-6	72	149	245	310	418	499
T-15-7	60	145	258	317	505	579
Average	63	134	229	308	529	642
Std. Dev.	11	24	29	89	107	126



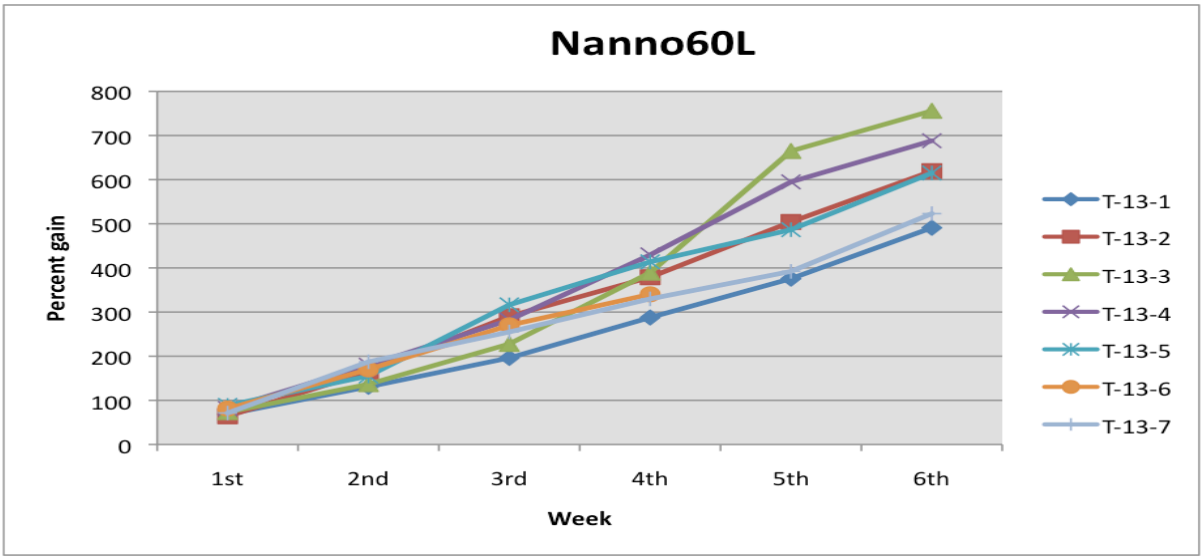
Appendix O. Weight of *L. vannamei* fed a diet of 60% *Nannochloropsis salina* (Nanno60L)

Algal Species: *Nannochloropsis salina* **Grid Position**: 13
Treatment: Nanno60L **Treatment #**: 8

Week 2	Week 3	Week 4	We
0.0575	0.0787	0.1010	0.1
0.0521	0.0844	0.1242	0.1
0.0487	0.0665	0.0921	0.1
0.0534	0.0822	0.1123	0.1
0.0577	0.0781	0.1274	0.1
0.0613	0.0913	0.1251	0.1
0.0567	0.0951	0.1175	0.1
0.0553	0.0823	0.1142	0.1
0.0042	0.0094	0.0134	0.0

T-13. Nanno60L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-13-1	69	131	196	288	376	491
T-13-2	64	165	291	379	504	619
T-13-3	73	137	228	389	665	756
T-13-4	82	180	282	430	595	688
T-13-5	89	155	316	414	487	615
T-13-6	81	170	270	340		
T-13-7	71	187	255	330	392	523
Average	76	161	263	367	503	615
Std. Dev.	9	21	40	50	113	99



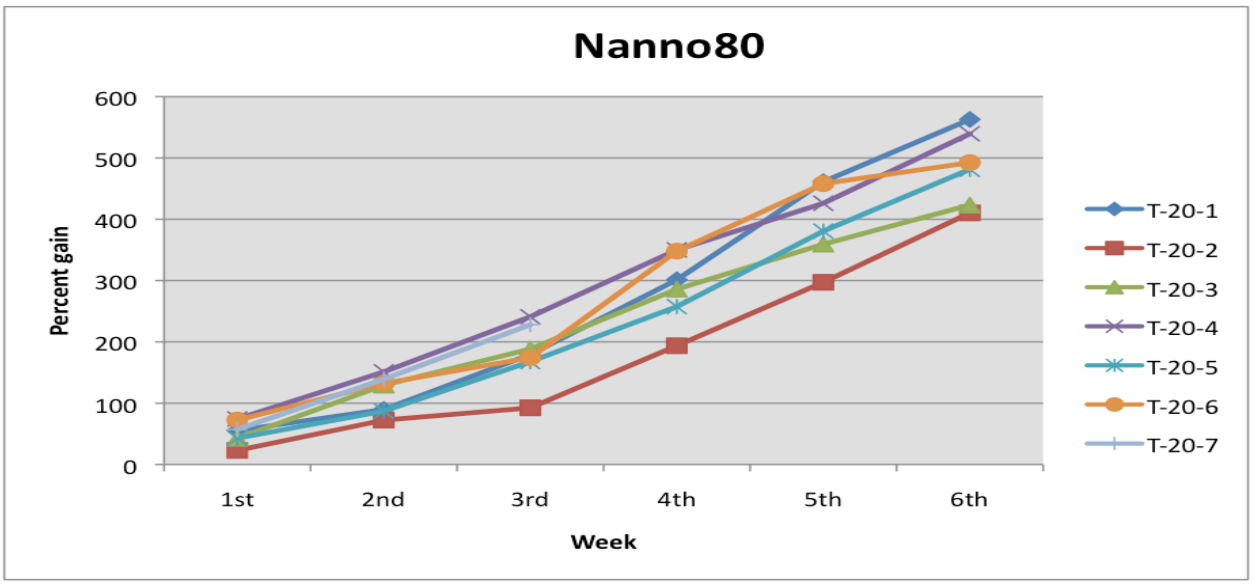
Appendix P. Weight of *L. vannamei* fed a diet of 80% *Nannochloropsis salina* (Nanno80)

Algal Species: *Nannochloropsis salina* **Grid Position**: 20
Treatment: Nanno80 **Treatment #**: 9

Week 2	Week 3	Week 4	We
0.0485	0.0592	0.0872	0.1
0.0391	0.0548	0.0612	0.0
0.0418	0.0672	0.0845	0.1
0.0481	0.0693	0.0941	0.1
0.0487	0.0638	0.0913	0.1
0.0571	0.0769	0.0908	0.1
0.0421	0.0641	0.0879	
0.0465	0.0650	0.0853	0.1
0.0061	0.0071	0.0111	0.0

T-20. Nanno80 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-20-1	55	90	179	301	461	563
T-20-2	23	72	92	194	297	410
T-20-3	43	129	188	286	359	423
T-20-4	74	151	241	350	426	539
T-20-5	43	87	168	257	381	482
T-20-6	73	132	174	348	458	492
T-20-7	57	139	228			
Average	53	114	182	289	397	485
Std. Dev.	18	31	48	59	64	61



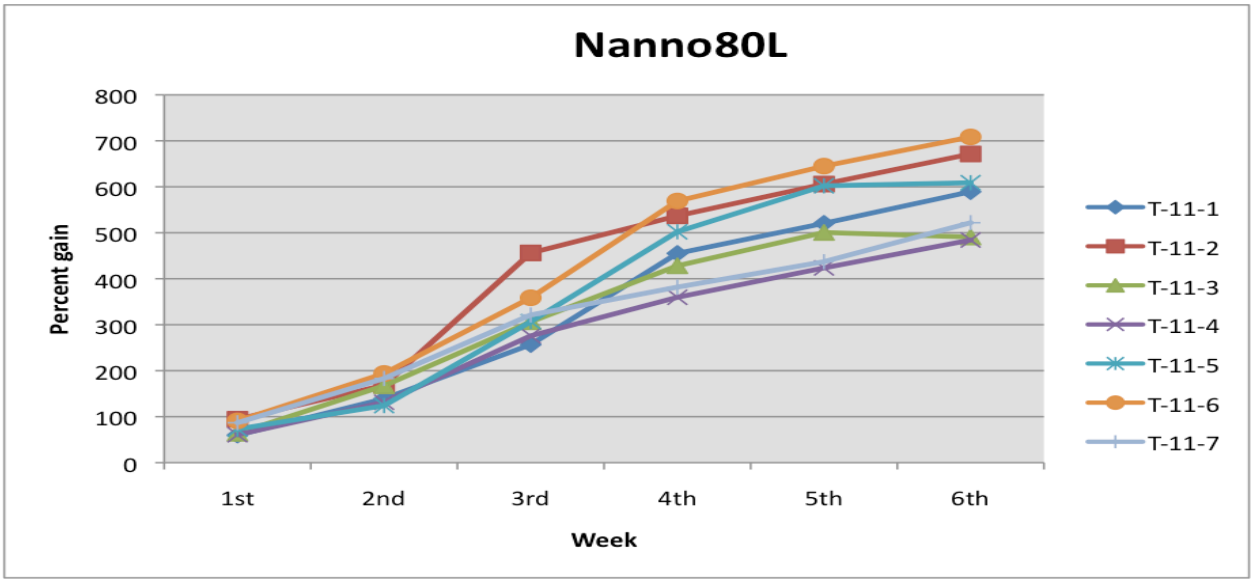
Appendix Q. Weight of *L. vannamei* fed a diet of 80% *Nannochloropsis salina* (Nanno80L)

Algal Species: *Nannochloropsis salina* **Grid Position**: 11
Treatment: Nanno80L **Treatment #**: 10

Week 2	Week 3	Week 4	We
0.0467	0.0698	0.1042	0.1
0.0523	0.0722	0.1496	0.1
0.0511	0.0834	0.1268	0.1
0.0498	0.0717	0.1165	0.1
0.0488	0.0632	0.1147	0.1
0.0475	0.0732	0.1142	0.1
0.0564	0.0855	0.1272	0.1
0.0504	0.0741	0.1219	0.1
0.0033	0.0078	0.0146	0.0

T-11. Nanno80L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-11-1	60	139	257	455	521	589
T-11-2	94	168	456	537	606	671
T-11-3	64	167	306	428	501	491
T-11-4	61	131	276	360	424	484
T-11-5	73	124	307	502	602	609
T-11-6	91	194	359	569	645	708
T-11-7	87	183	321	382	437	522
Average	76	158	326	462	534	582
Std. Dev.	15	27	66	78	87	88



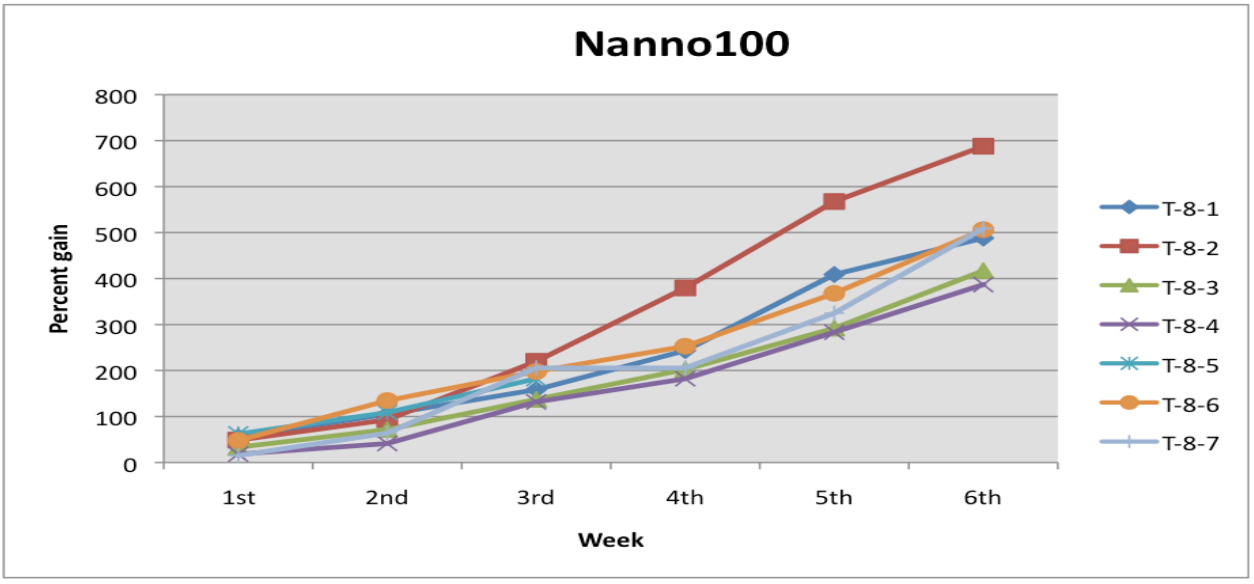
Appendix R. Weight of *L. vannamei* fed a diet of 100% *Nannochloropsis salina* (Nanno100)

Algal Species: *Nannochloropsis salina* **Grid Position**: 8
Treatment: Nanno100 **Treatment #**: 11

Week 2	Week 3	Week 4	We
0.0442	0.0582	0.0734	0.0
0.0394	0.0511	0.0848	0.1
0.0418	0.0539	0.0745	0.0
0.0398	0.0476	0.0781	0.0
0.0452	0.0582	0.0788	
0.0423	0.0671	0.0852	0.1
0.0367	0.0523	0.0975	0.0
0.0413	0.0555	0.0818	0.1
0.0029	0.0064	0.0083	0.0

T-8. Nanno100 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-8-1	56	105	158	243	409	488
T-8-2	49	93	220	380	568	688
T-8-3	33	72	137	202	292	417
T-8-4	18	41	132	182	283	387
T-8-5	62	109	182			
T-8-6	48	135	198	253	368	506
T-8-7	15	64	206	206	325	509
Average	40	88	176	244	374	499
Std. Dev.	18	31	34	71	106	105



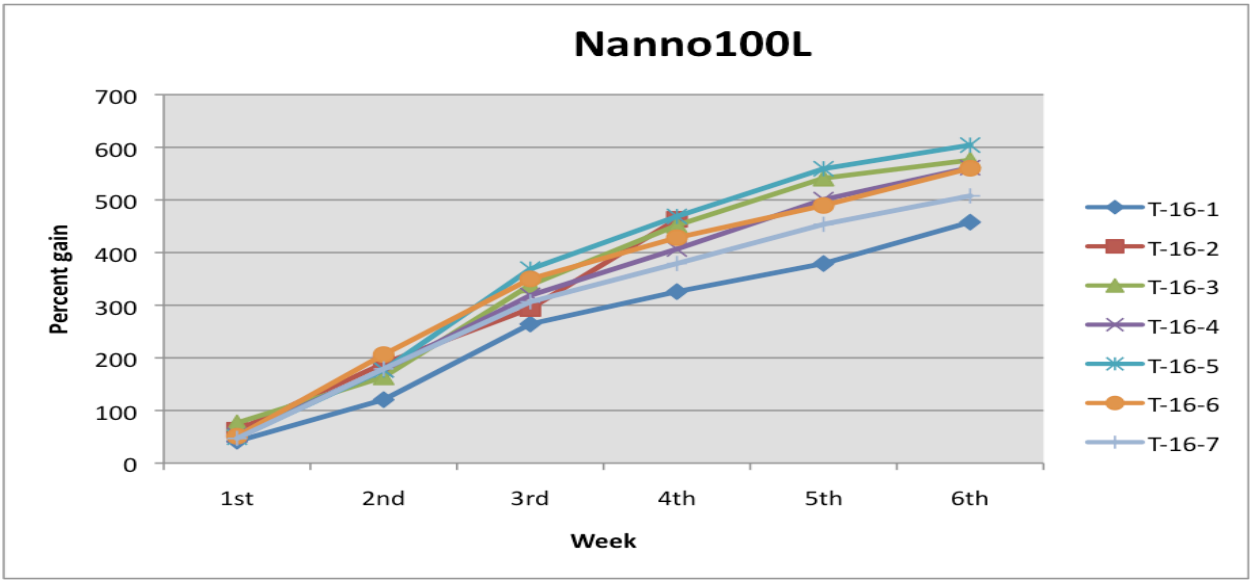
Appendix S. Weight of *L. vannamei* fed a diet of 100% *Nannochloropsis salina* (Nanno100L)

Algal Species: *Nannochloropsis salina* **Grid Position**: 16
Treatment: Nanno100L **Treatment #**: 12

Week 2	Week 3	Week 4	We
0.0483	0.0752	0.1242	0.1
0.0515	0.0917	0.1248	0.1
0.0487	0.0728	0.1210	0.1
0.0451	0.0818	0.1235	0.1
0.0428	0.0795	0.1345	0.1
0.0417	0.0845	0.1242	0.1
0.0438	0.0832	0.1211	0.1
0.0460	0.0812	0.1248	0.1
0.0036	0.0063	0.0046	0.0

T-16. Nanno100L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-16-1	42	121	264	326	379	458
T-16-2	62	189	294	463		
T-16-3	76	164	338	452	541	576
T-16-4	53	177	319	407	501	561
T-16-5	49	177	369	469	559	604
T-16-6	51	206	350	428	489	560
T-16-7	47	179	306	379	454	508
Average	54	173	320	418	487	544
Std. Dev.	12	27	36	52	65	53



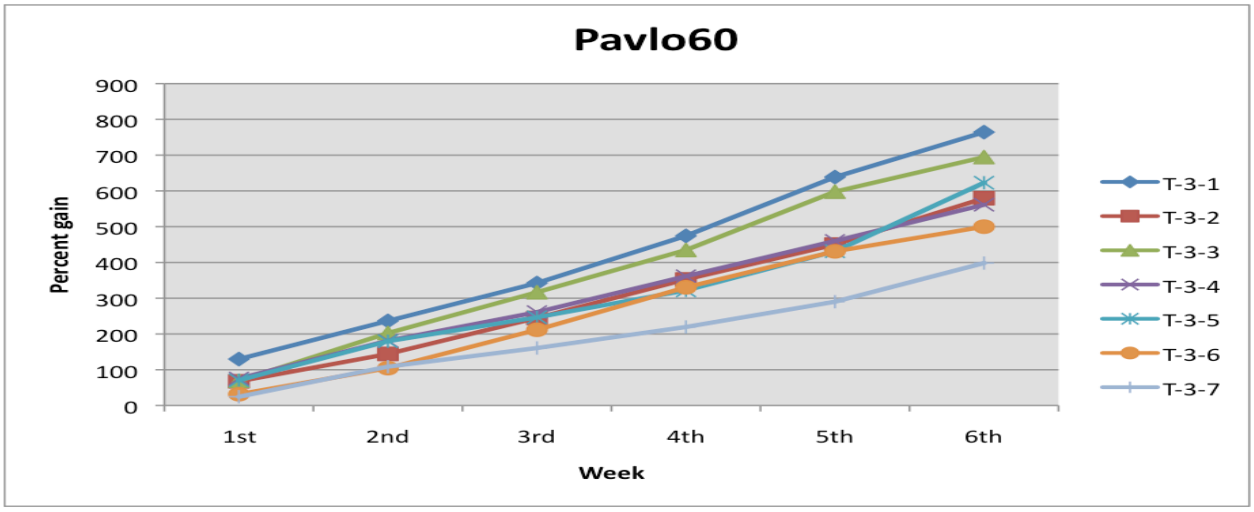
Appendix T. Weight of *L. vannamei* fed a diet of 60% *Pavlova sp.* (Pavlo60)

Algal Species: *Pavlova sp* **Grid Position**: 3
Treatment: Pavlo60 **Treatment #**: 13

Week 2	Week 3	Week 4	We
0.0547	0.0801	0.1053	0.1
0.0523	0.0762	0.1076	0.1
0.0467	0.0835	0.1150	0.1
0.0523	0.0838	0.1076	0.1
0.0531	0.0870	0.1078	0.1
0.0437	0.0679	0.1035	0.1
0.0445	0.0745	0.0931	0.1
0.0496	0.0790	0.1057	0.1
0.0045	0.0066	0.0066	0.0

T-3. Pavlo60 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-3-1	130	237	342	474	639	764
T-3-2	68	144	245	353	450	579
T-3-3	69	203	317	435	598	695
T-3-4	76	181	261	361	460	561
T-3-5	71	180	247	322	431	623
T-3-6	32	105	212	330	431	500
T-3-7	25	109	161	220	290	398
Average	67	165	255	356	471	589
Std. Dv.	34	49	61	82	116	121



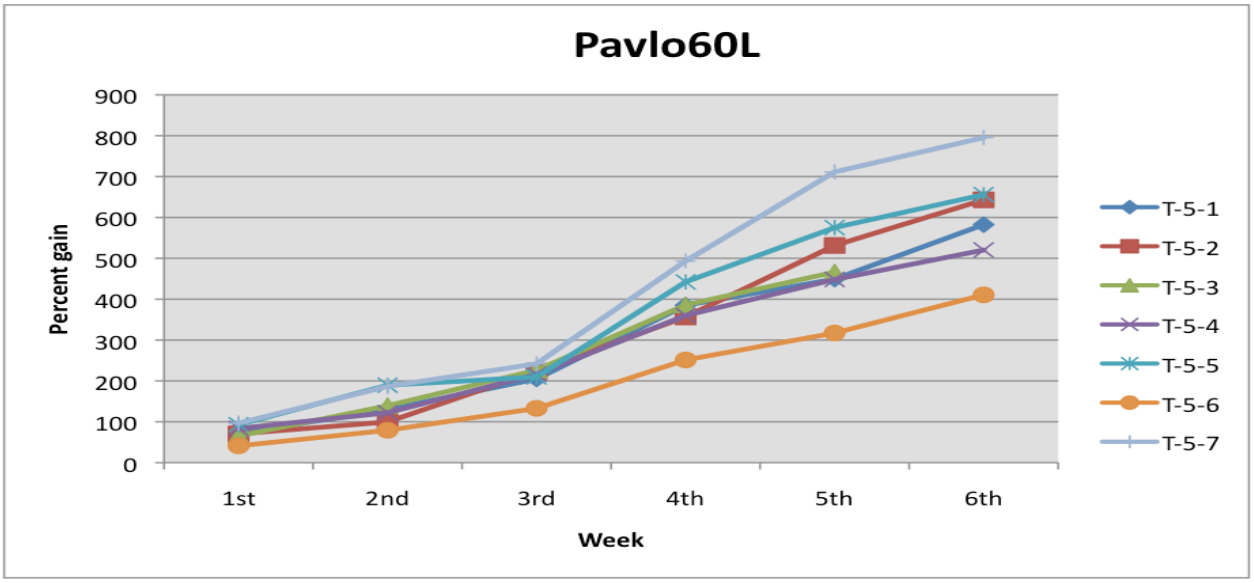
Appendix U. Weight of *L. vannamei* fed a diet of 60% *Pavlova sp*. (Pavlo60L)

Algal Species: *Pavlova sp.* **Grid Position**: 5
Treatment ID: Pavlo60L **Treatment #**: 14

Week 2	Week 3	Week 4	We
0.0534	0.0721	0.0954	0.1
0.0467	0.0547	0.0888	0.1
0.0529	0.0766	0.1044	0.1
0.0571	0.0691	0.0983	0.1
0.0511	0.0767	0.0821	0.1
0.0481	0.0611	0.0794	0.1
0.0468	0.0682	0.0814	0.1
0.0509	0.0684	0.0900	0.1
0.0039	0.0081	0.0096	0.0

T-5. Pavlo60L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-5-1	71	130	205	385	449	582
T-5-2	70	100	224	357	531	643
T-5-3	65	139	226	386	466	
T-5-4	84	122	216	360	448	520
T-5-5	93	189	210	442	575	656
T-5-6	41	79	133	251	317	411
T-5-7	97	187	242	493	711	795
Average	74	135	208	382	500	601
Std. Dev.	19	41	35	76	123	131



Appendix V. Weight of *L. vannamei* fed a diet of 80% *Pavlova sp*. (Pavlo80)

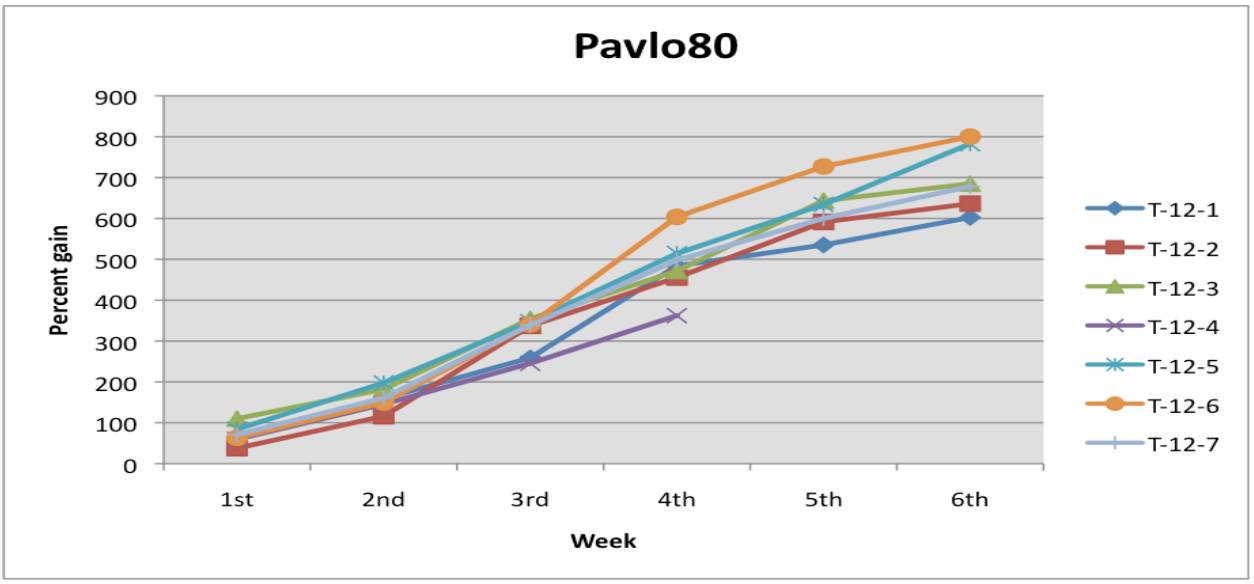
Algal Species: *Pavlova sp.* **Grid Position**: 12
Treatment ID: Pavlo80 **Treatment #**: 15

T-12. Pavlo80 – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-12-1	0.0287	0.0476	0.0745	0.1030	0.1676	0.1823	0.2016
T-12-2	0.0318	0.0438	0.0687	0.1390	0.1766	0.2199	0.2341
T-12-3	0.0301	0.0632	0.0849	0.1367	0.1720	0.2237	0.2363
T-12-4	0.0341	0.0544	0.0837	0.1178	0.1576		
T-12-5	0.0284	0.0523	0.0845	0.1270	0.1744	0.2085	0.2507
T-12-6	0.0266	0.0434	0.0663	0.1172	0.1872	0.2200	0.2394
T-12-7	0.0311	0.0532	0.0811	0.1365	0.1860	0.2177	0.2421
Average	0.0301	0.0511	0.0777	0.1253	0.1745	0.2120	0.2340
Std. Dev.	0.0025	0.0069	0.0078	0.0133	0.0103	0.0154	0.0169

T-12. Pavlo80 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-12-1	66	160	259	484	535	602
T-12-2	38	116	337	455	592	636
T-12-3	110	182	354	471	643	685
T-12-4	60	145	245	362		
T-12-5	84	198	347	514	634	783
T-12-6	63	149	341	604	727	800
T-12-7	71	161	339	498	600	678
Average	70	159	317	484	622	697
Std. Dev.	22	26	45	72	64	79



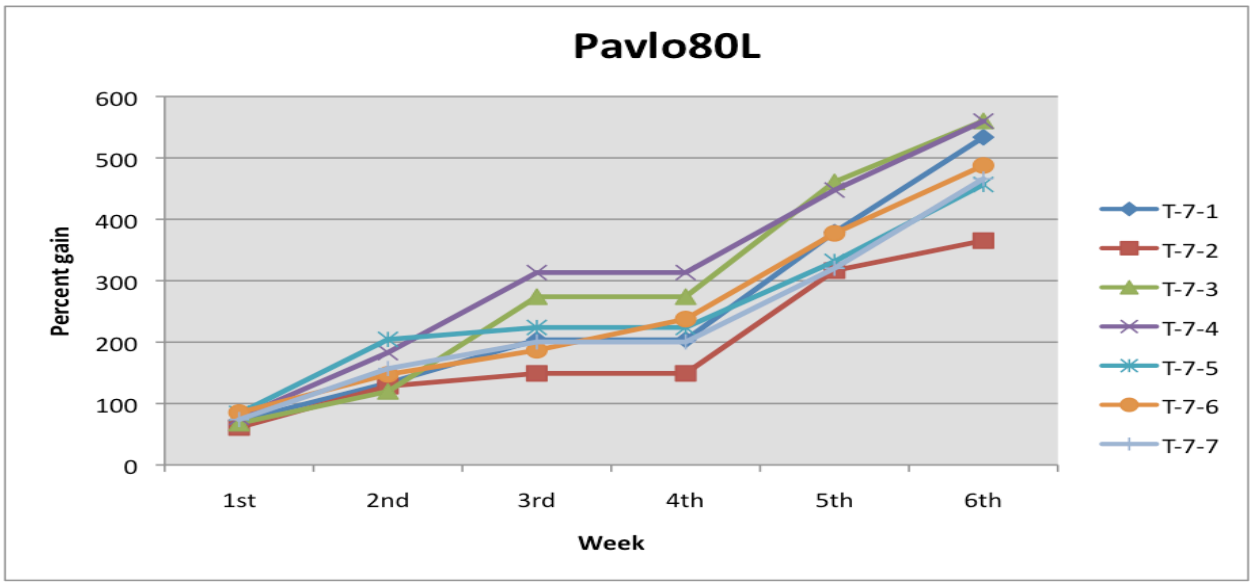
Appendix W. Weight of *L. vannamei* fed a diet of 80% *Pavlova sp.* (Pavlo80L)

Algal Species: *Pavlova sp.* **Grid Position**: 7
Treatment ID: Pavlo80L **Treatment #**: 16

Week 2	Week 3	Week 4	We
0.0547	0.0742	0.0966	0.0
0.0528	0.0749	0.0817	0.0
0.0477	0.0624	0.1062	0.1
0.0522	0.0832	0.1215	0.1
0.0539	0.0892	0.0949	0.0
0.0632	0.0844	0.0978	0.1
0.0541	0.0799	0.0934	0.0
0.0541	0.0783	0.0989	0.1
0.0046	0.0088	0.0123	0.0

T-7. Pavlo80L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-7-1	72	133	204	204	379	534
T-7-2	61	128	149	149	316	365
T-7-3	68	120	274	274	461	560
T-7-4	78	183	313	313	448	560
T-7-5	84	204	224	224	331	457
T-7-6	85	148	187	238	377	488
T-7-7	74	157	200	200	320	466
Average	75	153	222	229	376	490
Std. Dev.	8.6	30.8	55.4	53	59	69



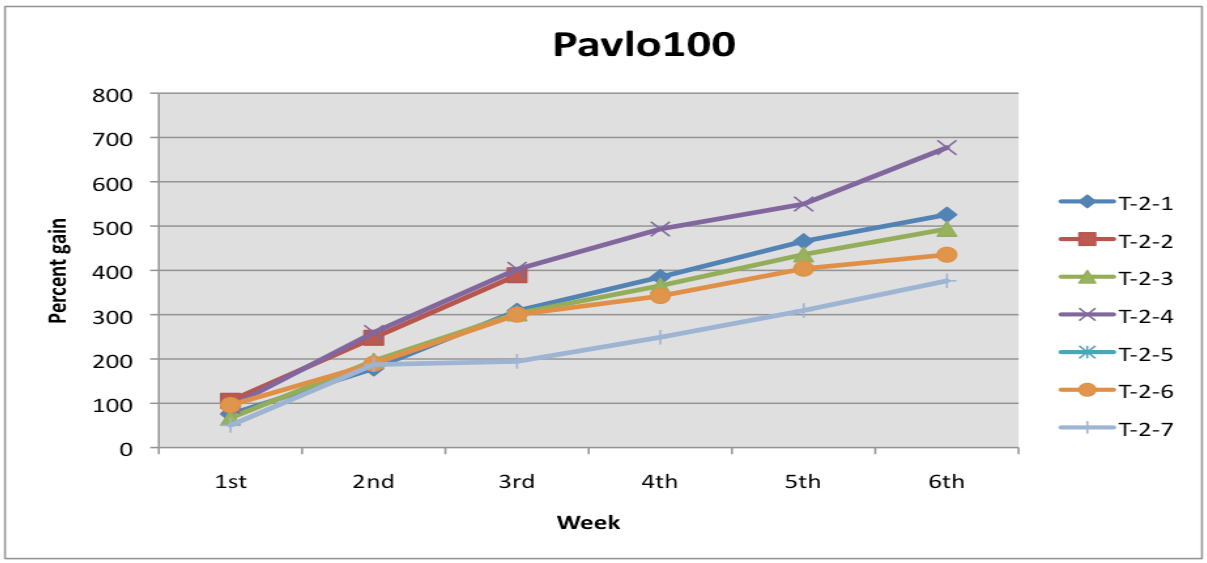
Appendix X. Weight of *L. vannamei* fed a diet of 100% *Pavlova sp.* (Pavlo100)

Algal Species: *Pavlova sp.* **Grid Position**: 2
Treatment ID: Pavlo100 **Treatment #**: 17

Week 2	Week 3	Week 4	W
0.0523	0.0830	0.1218	0
0.0434	0.0734	0.1032	
0.0521	0.0923	0.1257	0
0.0465	0.0867	0.1211	0
0.0670	0.0990	0.1368	0
0.0521	0.0997	0.1022	0
0.0522	0.0890	0.1185	0
0.0081	0.0101	0.0134	0

T-2. Pavlo100 – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-2-1	76	179	309	385	466	526
T-2-2	106	248	389			
T-2-3	67	196	303	365	436	494
T-2-4	93	260	402	493	550	677
T-2-5						
T-2-6	96	189	300	342	404	435
T-2-7	50	187	195	249	310	376
Average	81	210	316	367	433	502
Std. Dev.	21	35	75	88	88	114



Appendix Y. Weight of *L. vannamei* fed a diet of 100% *Pavlova sp*. (Pavlo100L)

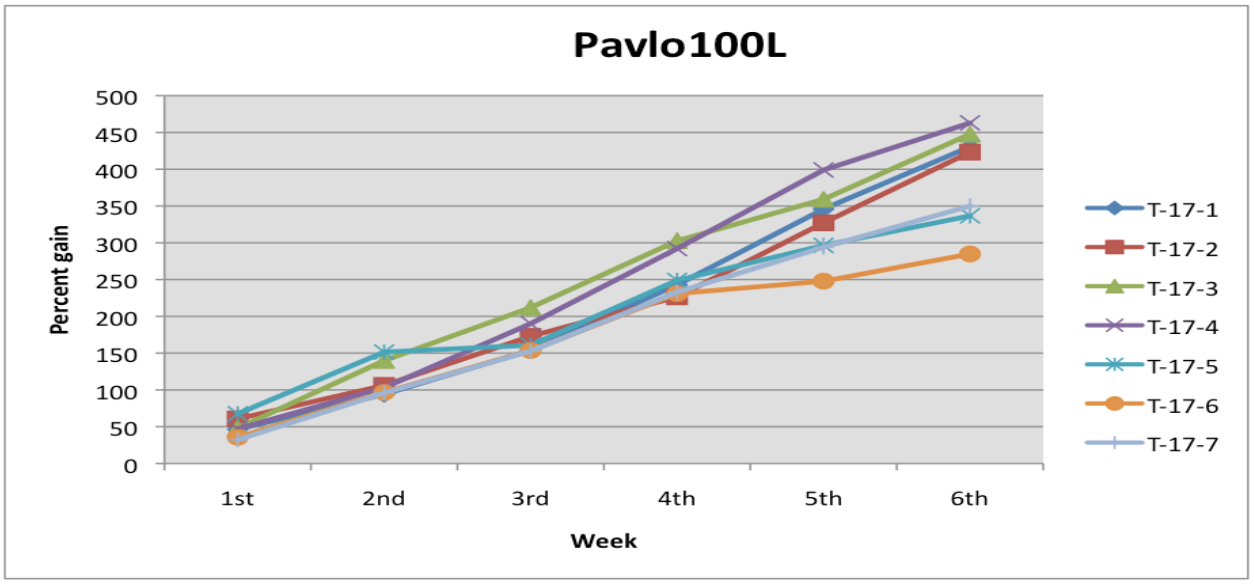
Algal Species: *Pavlova sp.* **Grid Position**: 17
Treatment ID: Pavlo100L **Treatment** #: 18

T-17. Pavlo100L – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-17-1	0.0334	0.0489	0.0648	0.0848	0.1145	0.1489	0.1772
T-17-2	0.0310	0.0498	0.0639	0.0846	0.1012	0.1324	0.1622
T-17-3	0.0296	0.0438	0.0710	0.0923	0.1192	0.1359	0.1621
T-17-4	0.0291	0.0428	0.0591	0.0844	0.1141	0.1452	0.1638
T-17-5	0.0311	0.0521	0.0782	0.0811	0.1085	0.1233	0.1358
T-17-6	0.0362	0.0491	0.0712	0.0918	0.1198	0.1259	0.1393
T-17-7	0.0316	0.0418	0.0619	0.0799	0.1052	0.1245	0.1422
Average	0.0317	0.0469	0.0672	0.0856	0.1118	0.1337	0.1547
Std. Dev.	0.0024	0.0040	0.0066	0.0048	0.0070	0.0102	0.0155

T-17. Pavlo100L – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-17-1	46	94	154	243	346	431
T-17-2	61	106	173	226	327	423
T-17-3	48	140	212	303	359	448
T-17-4	47	103	190	292	399	463
T-17-5	68	151	161	249	296	337
T-17-6	36	97	154	231	248	285
T-17-7	32	96	153	233	294	350
Average	48	112	171	254	324	391
Std. Dev.	13	23	23	31	50	67



Appendix Z. Weight of *L. vannamei* fed a Control diet

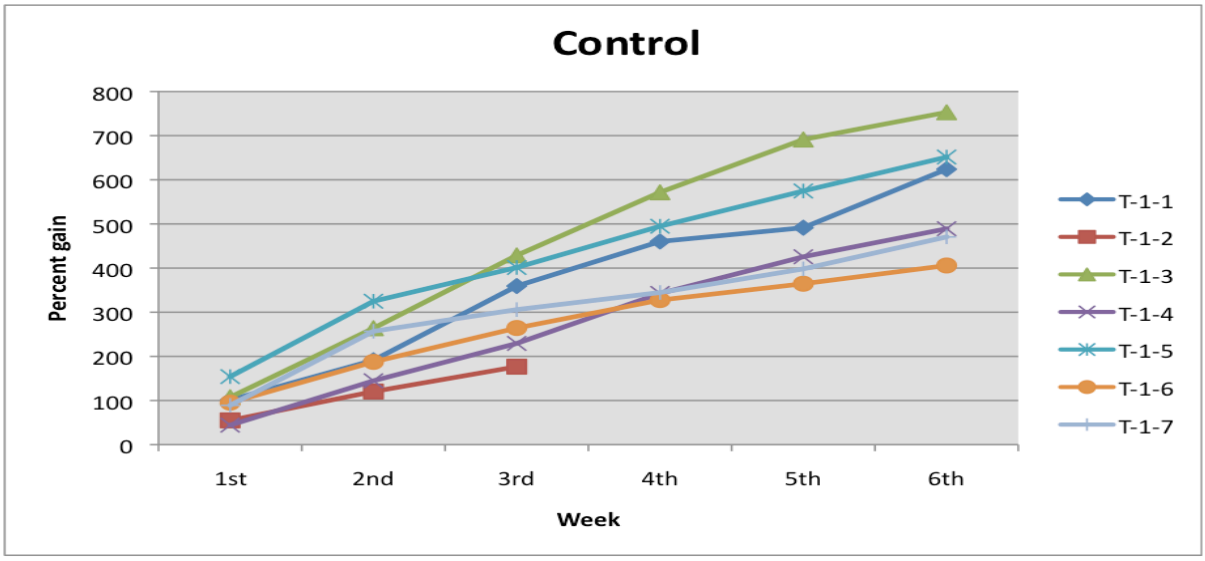
Algal Species: *n/a* **Grid Position**: 1
Treatment ID: Control **Treatment** #: 19

T-1. Control – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-1-1	0.0317	0.0638	0.0923	0.1456	0.1776	0.1875	0.2295
T-1-2	0.0369	0.0572	0.0813	0.1021			
T-1-3	0.0240	0.0498	0.0873	0.1269	0.1612	0.1899	0.2047
T-1-4	0.0378	0.0545	0.0925	0.1245	0.1673	0.1987	0.2227
T-1-5	0.0229	0.0582	0.0973	0.1148	0.1362	0.1545	0.1721
T-1-6	0.0392	0.0765	0.1127	0.1428	0.1676	0.1821	0.1983
T-1-7	0.0385	0.0728	0.1374	0.1563	0.1712	0.1918	0.2197
Average	0.0330	0.0618	0.1001	0.1304	0.1635	0.1841	0.2078
Std. Dev.	0.0070	0.0098	0.0191	0.0189	0.0144	0.0155	0.0210

T-1. Control – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-1-1	101	191	359	460	491	624
T-1-2	55	120	177			
T-1-3	108	264	429	572	691	753
T-1-4	44	145	229	343	426	489
T-1-5	154	325	401	495	575	652
T-1-6	95	188	264	328	365	406
T-1-7	89	257	306	345	398	471
Average	87	213	309	424	491	566
Std. Dev.	40	72	92	100	123	131



Appendix AA. Weight of *L. vannamei* fed a Basal diet

Algal Species: *n/a - Basal* **Grid Position**: 14

Treatment ID: Basal **Treatment #**: 20

T-14. Basal – Weight Per Animal (g)

Replicate	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
T-14-1	0.0249	0.0577	0.0934	0.1263	0.1532	0.1982	0.2174
T-14-2	0.0331	0.0628	0.0922	0.1133	0.1348	0.1968	0.2224
T-14-3	0.0276	0.0511	0.0853	0.1288	0.1567	0.1993	0.2239
T-14-4	0.0298	0.0500	0.0929	0.1318	0.1623	0.1856	0.2178
T-14-5	0.0287	0.0498	0.0832	0.1235	0.1324	0.1573	0.1762
T-14-6	0.0262	0.0540	0.0854	0.1275	0.1572	0.1925	0.2052
T-14-7	0.0309	0.0622	0.0961	0.1366	0.1589	0.1823	0.2241
Average	0.0287	0.0554	0.0898	0.1268	0.1508	0.1874	0.2124
Std. Dev.	0.0028	0.0056	0.0050	0.0073	0.0121	0.0148	0.0173

T-14. Basal – Weight Gain (%)

Replicate	1st	2nd	3rd	4th	5th	6th
T-14-1	132	275	407	515	696	773
T-14-2	90	179	242	307	495	572
T-14-3	85	209	367	468	622	711
T-14-4	68	212	342	445	523	631
T-14-5	74	190	330	361	448	514
T-14-6	106	226	387	500	635	683
T-14-7	101	211	342	414	490	625
Average	94	214	345	430	558	644
Std. Dev.	22	31	53	75	92	87