NUTRITIONAL CONTRIBUTION OF PHYTOPLANKTON TO THE PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI*

A Dissertation

by

DAGOBERTO RAUL SANCHEZ CORRALES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Nutrition

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Approved by:

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ABSTRACT

Nutritional Contribution of Phytoplankton to the Pacific White Shrimp *Litopenaeus vannamei*. (May 2012)

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Co-Chairs of Advisory Committee: Dr. Delbert Gatlin III
Dr. Addison L. Lawrence

The goal of this study was to characterize the nutritional contribution of microalgae to white-legged shrimp and optimize fish meal (FM) and fish oil (FO) inclusion levels in their diets in the presence of microalgae. Phytoplankton composition was first determined in a typical Peruvian intensive commercial shrimp farm and in a semi-closed greenhouse-covered reservoir. A predominance of 76.3% cyanobacteria was observed for most of 9 months in all shrimp ponds. However, with the fertilization program in a reservoir tank, 60.7% diatoms and 22.8% cyanobacteria predominated. Thus, with the imposed fertilization regimen, the microalgae composition was manipulated to be different than that in commercial shrimp ponds. The microalgae composition was then evaluated along with different dietary levels of FM and squid meal (SM) in a feeding trial to evaluate the potential of phytoplankton to reduce FM and SM levels in shrimp feeds. Six diets were formulated to contain either 5, 10 or 20% SM combined with either 6.5 or 12% FM. Dietary effects on growth and survival were compared in a "clear-water system" (CWS) and a "green-water system" (GWS). Results suggest that 6.5% FM and 5% SM can be used as a cost-effective combination in feeds for shrimp.

The effects of different dietary levels of FO and soybean lecithin (LT) on shrimp growth in CWS and GWS were evaluated in another feeding trial to determine if dietary phospholipids and phytoplankton increase the availability of essential fatty acids (EFAs) to shrimp. Six diets were formulated to contain 1, 2 or 3% FO combined with either 1 or 4% LT. Shrimp fed diets containing 1% LT and 1% FO in both systems had significantly lower weight gain and higher feed conversion ratio. Cephalothorax lipids and phospholipids were higher in shrimp fed diets containing 4% LT. Inclusion of 4% LT increased the availability of EFAs, and could contribute to reduce the FO in shrimp diets. The contribution of phytoplankton to shrimp weight gain, varied from 38.8 to 60.6%.

This study demonstrated that cost-effective diets could be formulated with reduced inclusion levels of FM and FO considering the contribution of microalgae to the nutrition of shrimp.

DEDICATION

To my wife, daughter and son

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I would like to thank my committee chair, Dr. Delbert Gatlin III, my co-chair Dr. Addison Lawrence and committee members, Drs. Joe Fox and Christopher Bailey for their guidance and support throughout the course of this research.

Thanks also to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience. I also want to extend my gratitude to the technicians of the AES for assistance in conducting the experiment.

Finally, thanks to my mother and father for their encouragement and to my wife for her love.

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CHAPTER I

INTRODUCTION

World production of farmed shrimp has grown 16% per year, since 1984. In 2009, shrimp aquaculture yields were estimated at 3.1 million metric tons (MMT) with total farmed shrimp landings valued at US$ 12.9 billion (FAO 2011). Until 2001, black tiger shrimp, *Penaeus monodon*, was the main species cultured comprising 70% of world production (FAO 2008). Widespread catastrophic disease in combination with the lack of a genetic improvement program to address disease resistance and improved growth motivated many Asian countries such as China, Thailand, Vietnam and Indonesia to begin culturing the Pacific white shrimp, *Litopenaeus vannamei*. In 2003, the production of *L. vannamei* exceeded black tiger shrimp by 309,172 MT (59% of world production - FAO 2011) and it is projected that for 2013, this species will represent about 72% of world production (Valderrama & Anderson 2011).

The formulation of cost-effective diets is the goal of all animal production industries. This is especially true for marine shrimp aquaculture, where phytoplankton play an important role in providing the base of the aquatic food chain for coastal production (Leber & Pruder 1988; Moss & Pruder 1995; Otoshi, Montgomery, Look & Moss 2001; Michele, Melony, Stuart, Sandy, Peter & Nigel 2004). *P. monodon* requires a higher protein level in its diet than *L. vannamei* (36–42% compared to 23–35%;

This dissertation follows the style of *Aquaculture Research.*

because it is assumed to obtain most of its nutrients from higher trophic levels, and domesticated *L. vannamei* more effectively utilizes proteins from plant origin (Gaxiola, Brito, Maldonado, Jimenez-Yan, Guzman, Leticia, Brito, Luis & Cuzon 2006), which results generally in lower feed costs.

White shrimp, *Litopenaeus vannamei*, is cultured in extensive, semi-intensive, and intensive systems (Frias-Espericueta, Harfush-Melendez & Paez-Osuna 2000). According to Tacon, Hasan & Subasinghe (2006) in 1999, 82% of the world shrimp farms used extensive and semi-intensive pond-based grow-out culture systems. Microalgae which comprise important parts of the natural productivity in the various aquatic systems play a vital role in the rearing of aquatic animals (Muller-Feuga 2000). These microalgae also play an important role in the dynamics, (i.e., water quality, dissolved oxygen, ammonia, alkalinity and pH) of all shrimp pond systems. In extensive and semi-intensive pond-based culture systems (from 500 to ~6,000 kg ha^{-1} $crop^{-1}$), microalgae typically contribute greater to the food supply than in intensive culture systems (~7,000 to ~40,000 kg ha^{-1} $crop^{-1}$). Thus, with the higher shrimp biomass present in intensive systems there is a requirement for greater amounts of compounded feed with a higher nutrient density and aeration for optimal shrimp growth (D'Abramo & Conklin 1995). However, if the phytoplankton levels are limiting in shrimp pond production systems, it will have a negative effect on the quality of the culture water, due to the fact that the oxygen and carbon dioxide balance in the water column cannot be maintained. Furthermore, the lack of shading will increase the benthic algae levels. Thus, phytoplankton play a crucial role in shrimp ponds (Castille & Lawrence 1989;

Lawrence & Houston 1993; Tacon 1996) as it not only contributes nutrients but also stabilizes the culture system thereby optimizing production costs.

The value of microalgae to the nutrition of white shrimp in aquaculture has been documented to be substantial, and thus it is critical to more specifically determine the contribution of various essential nutrients from natural productivity to optimize diet formulas for shrimp (Tacon, Cody, Conquest, Divakaran, Forster & Decamp 2002; Ju, Forster & Dominy 2009). Microalgae species can vary significantly in their nutritional value and this may also change under different culture conditions (Enright, Newkirk, Craigie & Castell 1986; Brown, Jeffrey, Volkman & Dunstan 1997). Protein, carbohydrate, lipids, minerals and vitamins make up 90 – 95% of the dry weight of an algal cell (Brown, Jeffrey & Garland 1989). Microalgae grown to late-logarithmic growth phase typically contain 30 to 40% protein, 10 to 20% lipid and 5 to 15% carbohydrate (Brown *et al.* 1997; Renaud, Thinh & Parry 1999). The optimum nutritional value of microalgae, as aquaculture feed species, is very much influenced by the fatty acid composition of the lipids (Langdon & Walcock 1981; Chu & Webb 1984; Enright *et al.* 1986; Dunstan, Volkman, Jeffrey & Barret 1996) and, to a lesser extent, by the amino acid composition of the proteins (Enright *et al.* 1986; Brown & Jeffrey 1992) and the composition of the carbohydrates (Brown & Jeffrey 1992).

Shrimp aquaculture requires the use of artificial feeds in commercial pond production to supplement nutrients obtained by shrimp from natural productivity. Presently, 75 to 80% of all farmed shrimp are grown on commercial feeds (Deutsch, Gräslund, Folke, Troell, Huitric, Kautsky & Lebel 2007) and it is proposed that

commercial feeds will soon replace farm-made feeds in most shrimp farming (Tacon 2002). Marine fish meals are often utilized as a major protein source in these diets because they have high protein content**,** a suitable amino acid profile (particularly lysine and methionine), a desirable fatty acid (ω fatty acids) and phospholipids profile, as well as adequate mineral and vitamin levels, acceptable palatability, digestibility and even unknown nutritional factors (Tacon & Akiyama 1997; Sudaryono, Tsvetnenko & Evans 1999; Paripatananont, Boonyaratpalin, Pengseng & Chotipuntu 2001; Zaldivar 2002; Forster, Dominy, Obaldo & Tacon 2003; Yu 2003; Samocha, Davis, Saoud & DeBault 2004; Alvarez, Hernandez-Llamas, Galindo, Fraga, García & Villareal 2007). The levels of fish meal inclusion in commercial diets for shrimp have been reported to vary from 10% to 50% (Lim & Dominy 1990; Akiyama & Dominy 1991; Tacon 1993; Tacon & Barg 1998). Tacon (2004) reported a range of fish meal use for marine shrimp diets of 25 to 50% for starter feeds and 15 to 35% on grower feeds, establishing a use average of 26% (Tacon 2002), depending on factors such as protein quality, digestibility, percentage of protein in the diet, culture system, species of shrimp**,** animal husbandry methodology and percent contribution of natural productivity.

Increased demand for aquaculture feeds and relatively stable fish meal (FM) and fish oil (FO) production has led to its high demand, unreliable quality and a steady increase in price. It is estimated (Tacon & Metian 2008) that in 2006 the aquaculture sector consumed 3.72 MMT of FM and 0.83 MMT of FO, or the equivalent of 16.6 MMT of small pelagic forage fish (using a wet fish to FM processing yield of 22.5% and a wet fish to FO processing yield of 5%) with an overall ratio of wild fisheries inputs to

farmed fish outputs (fish-in to fish-out ratio) of 0.70. Moreover, coupled with the current estimated use of 5 to 7 MMT of trash fish as a direct food source for farmed fish, it is estimated that the aquaculture sector consumed the equivalent of 20–25 MMT of fish as feed in 2003 for the total production of about 30 MMT of farmed finfish and crustaceans (fed finfish and crustaceans 22.79 MMT and filter feeding finfish 7.04 MMT). This means that aquaculture has used up to 88.5% of the world's FO and 68.2% of the world's FM (Tacon & Forster 2001; Tacon & Barg 2001; Tacon & Metian 2008). This situation in the FM market despite high demand could lead to unpredictable aquaculture growth. Thus, research efforts are imperative, to identify and develop sustainable alternative sources of protein for aquatic diets, decreasing the inclusion levels of FM in shrimp feeds used for the commercial production of shrimp. This objective needs to be achieved in the short term, in order to assure a sustainable growth of the shrimp farming industry.

Due to the fact that increased production of sustainable FM and FO from natural sources seems unlikely and that according to Tacon & Metian (2008), 1.4 kg of wild fish (pelagic, wet-weight basis) is used to produce 1 kg of shrimp (e.g., FCR = 1.7, 4.5 kg pelagic fish = 1 kg FM, 18.5% FM inclusion rate), the aquaculture feed industry needs to gradually reduce the dependency on wild FM and FO and to develop new sources of marine protein and oil. Thus, it is imperative to develop viable commercially sustainable alternative protein sources to supply the growing aquafeeds industry (Lim & Dominy 1990; D'Abramo & Lovell 1991; Tacon 1993; Tidwell, Webster, Yancey & D'Abramo

1993; D'Abramo & Conklin 1995; Sudaryono, Hoxey, Kailis & Evans 1995; Naylor, Hardy, Bureau, Chiu, Elliott, Farrell, Forster, Gatlin, Goldburg, Hua & Nichols 2009).

The general goal of this study was to evaluate the contribution of phytoplankton to the nutrition of the Pacific white shrimp *Litopenaeus vannamei*, reared in Tumbes, Peru, replacing FM and FO with sustainable cost-effective alternative sources.

The specific objectives of the present study were: 1) to monitor primary productivity (phytoplankton composition) levels as a proxy for natural productivity in a typical Peruvian semi-closed intensive commercial farm in Tumbes; 2) to compare the findings with data from traditional marine aquaculture ponds; 3) to characterize the dynamics of the microalgae community and biomass through the measure of Secchi depth, dominant genera determination and chlorophyll composition in a controlled system; 4) to determine the weight gain and survival of *Litopenaeus vannamei* with different dietary levels of FM and squid meal in indoor tanks with and without microalgae and measure the relative contribution of microalgae; 5) to determine if the dietary PLs increase the availability of EFAs, and 6) evaluate if phytoplankton contributes to the dietary requirements of PLs and EFAs, optimizing FO inclusion levels in commercial shrimp diets.

CHAPTER II

CHARACTERIZATION OF THE PHYTOPLANKTON COMMUNITY IN A WHITE SHRIMP SEMI-CLOSED INTENSIVE SYSTEM AND A CONTROLLED ENVIRONMENT IN TUMBES, PERU

Introduction

Phytoplankton is a nutrient source for numerous aquacultured species, providing them with various nutrients including essential vitamins and polyunsaturated fatty acids (Muller-Feuga 2000). The role of phytoplankton as the primary trophic level of the aquatic food chain for coastal commercial pond production of marine shrimp has been well established (De Pauw & Persoone 1988; Paerl & Tucker 1995). These microalgae also significantly affect water quality, especially dissolved oxygen and pH, of shrimp pond systems (McIntosh, Fitzimmons, Collins & Stephens 2006; Velasquez, Cabrera, Rosas & Troccoli 2007). Phytoplankton plays a crucial role in extensive, semi-intensive and intensive commercial shrimp ponds (Castille & Lawrence 1989; Lawrence & Houston 1993; Tacon 1996). In extensive and semi-intensive pond-based grow-out culture systems (from 500 to 6,000 kg ha^{-1} $crop^{-1}$), water quality and food supply are more affected by the presence of microalgae than in intensive culture systems (7,000 – 34,000 kg ha^{-1} $crop^{-1}$) (Hunter, Pruder & Wyban 1987). This is largely due to the fact that in these systems the higher biomass requires greater amounts of supplemental feed and, as a result, pond management is substantially different (e.g., aerators to maintain required dissolved oxygen levels). However, in intensive systems, if there is a lack of

phytoplankton, benthic algae will grow, having also a negative effect on the quality of the culture water. This is largely due to the fact that the oxygen, carbon dioxide and nitrogen balance in the water column cannot be maintained (Briggs & Funge-Smith 1994). Furthermore, aeration demand increases and large rations of nutritionally complete feed are required, causing potential economic losses, as production costs increase (Kuban, Lawrence & Wilkenfield 1985).

Intensive shrimp pond systems in Tumbes, Peru, culture the white-legged shrimp, *Litopenaeus vannamei*. It is also the major species used for aquaculture in the rest of the western hemisphere. Culture ponds are typically high-density polyethylene (HDPE)-lined and lack bottom soil contact with water. Water is generally treated with chlorine in the pond before stocking, thus there is a limited amount of phytoplankton in the water column of the ponds. Unlike earthen ponds, secondary productivity is not very significant. Further, the contribution of natural productivity to the dietary requirements of shrimp decreases as the shrimp biomass per square meter increases in the pond. The objective of this study was to determine phytoplankton species and levels in a semi-closed intensive shrimp farm in Tumbes, Peru and compare it to a controlled system enclosed in the same farm. This research will provide information to understand the dynamics of microalgae in the water column and optimize management protocols in intensive shrimp ponds.

Materials and Methods

Research site

Research for this study was conducted at the semi-closed intensive shrimp farm, LATIMAR, and the Aquatic Experimental Center (AEC) of Alicorp in Tumbes, on the northern Pacific coast of Peru (latitude 3°27'33.22"S, longitude 80°19'54.13"W – 3msl). This farm stocks the white-legged shrimp (*Litopenaeus vannamei*) and is a typical Peruvian semi-closed intensive commercial farm, comprised of 12 high-density polyethylene (HDPE) lined, sediment-free, greenhouse enclosed, square and rectangular shaped, 1-ha ponds (Fig. 2.1).

Prior to stocking, ponds were filled with water pumped from the "El Venado" estuary. This water was subsequently chlorinated (hypochloric acid) in order to eliminate all possible pathogens from the seawater added to the ponds. This procedure also eliminated the phytoplankton and zooplankton in the seawater. Pond water was aerated for 24 h in order to dissipate chlorine prior to stocking ponds at a density of 85 postlarvae per square meter (PL_{10-12}). Ponds were greenhouse-enclosed, maintaining water temperature at 29 – 33 °C. Ponds had rounded corners and were 1.2-m deep, containing a central drain for siphoning of organic material, uneaten feed, dead natural productivity and animal waste produced during the production phase. Aeration and circulation was provided by 17 paddle wheel aerators at a rate of 34 hp per ha. Shrimp were fed a commercial 40% crude protein (CP) 2.5 x 5 mm feed (Nicovita®, Alicorp, Lima, Peru) for the first 30 days of culture, then a 35% CP feed (Nicovita®, Alicorp, Lima, Peru) for the next 60 days and finished with a 30% CP feed (Nicovita®, Alicorp,

Lima, Peru). Feed was broadcast four times per day (0700, 1100, 1500, and 1900 h). The feeding rate was 0.2 - 0.5 g of feed per shrimp per day depending upon size of shrimp and consumption rate as determined by six sampling trays per pond. Samples comprised 3 - 4% of the total feed broadcast per ration and were collected from the trays after 2 h.

Pond fertilization was undertaken with an initial dose of 200 kg/ha of commercial organic fertilizer. This was followed by 50 kg/ha each day for three consecutive days using Nutrilake® a commercial inorganic fertilizer containing 15% nitrogen as $N\text{-}NO_3$, 6% phosphorus as P_2O_5, 23.2% sodium, 3.5% silicate as SiO_2, 0.35% boron, 0.15% magnesium, 0.08% sulfur and 0.37% potassium, for establishment of a phytoplankton bloom prior to stocking. Molasses carbohydrate was balanced with feed nitrogen during the first 21 days after stocking to maintain in water a C/N ratio of ten. After 21 days, 120 - 150 kg of molasses^{-1} ha^{-1} d^{-1} was added to the pond water until harvest. If pond water levels of cyanobacteria exceeded 200,000 c mL^{-1}, the dose of molasses was increased to 300 kg ha^{-1} d^{-1}. If levels of cyanobacteria continued to remain high, 200 kg ha^{-1} calcium hydroxide was added to the pond water and/or fresh well water was added to ponds at an exchange rate of 10 - 15% per day. Water exchange in all ponds typically averaged 10% per day over the 9-month production season.

Description of reservoir system

The AEC reservoir system, located within LATIMAR (Fig. 2.1), consisted of six wooden, HDPE-lined, sediment-free, greenhouse-enclosed tanks (volume: 28.27 m^3). Tanks contained water extracted from the “El Venado” estuary, used as a water source by LATIMAR, and aerated by a 2-hp blower. In order to standardize measurements and

characterize the microalgae community of the system, one of the reservoirs was provided with continuous light from four evenly-distributed groups of ten fluorescent lights (36 W, 2,500 lx at 40 cm over the water surface). This regime was adopted to enhance growth of microalgae.

Characterization of phytoplankton in ponds

Weekly samples of phytoplankton were taken from the ponds from November 2006 to July 2007. Samples were collected at 12:00 hours from a depth of 30 cm using 1-L plastic bottles. Subsamples (~150-mL) were preserved by adding 2 mL of Lugol's solution (Throndsen 1978). Cells were allowed to settle for 10 h and subsequently processed for taxonomic and cell count analysis using an improved Neubauer hemocytometer at 600x magnification. Methodology for cell counts followed that of Venrick (1978). Malca's (1997) manual was used to separate microalgae into taxonomic divisions for subsequent data analysis. In addition, water temperature, dissolved oxygen (DO), pH and nitrite (NO_2-N) were monitored for all 12 ponds twice daily and within a 1-h period at 06:00 and 18:00 h using a YSI 85® meter (Yellow Spring Instruments, Yellow Springs, Ohio, USA), a YSI pH10 Ecosense® pen-style meter and by preparing a 10-mL sample for photometric analysis using a YSI 9000® photometer. Salinity was measured by refractometer for each pond on a weekly basis.

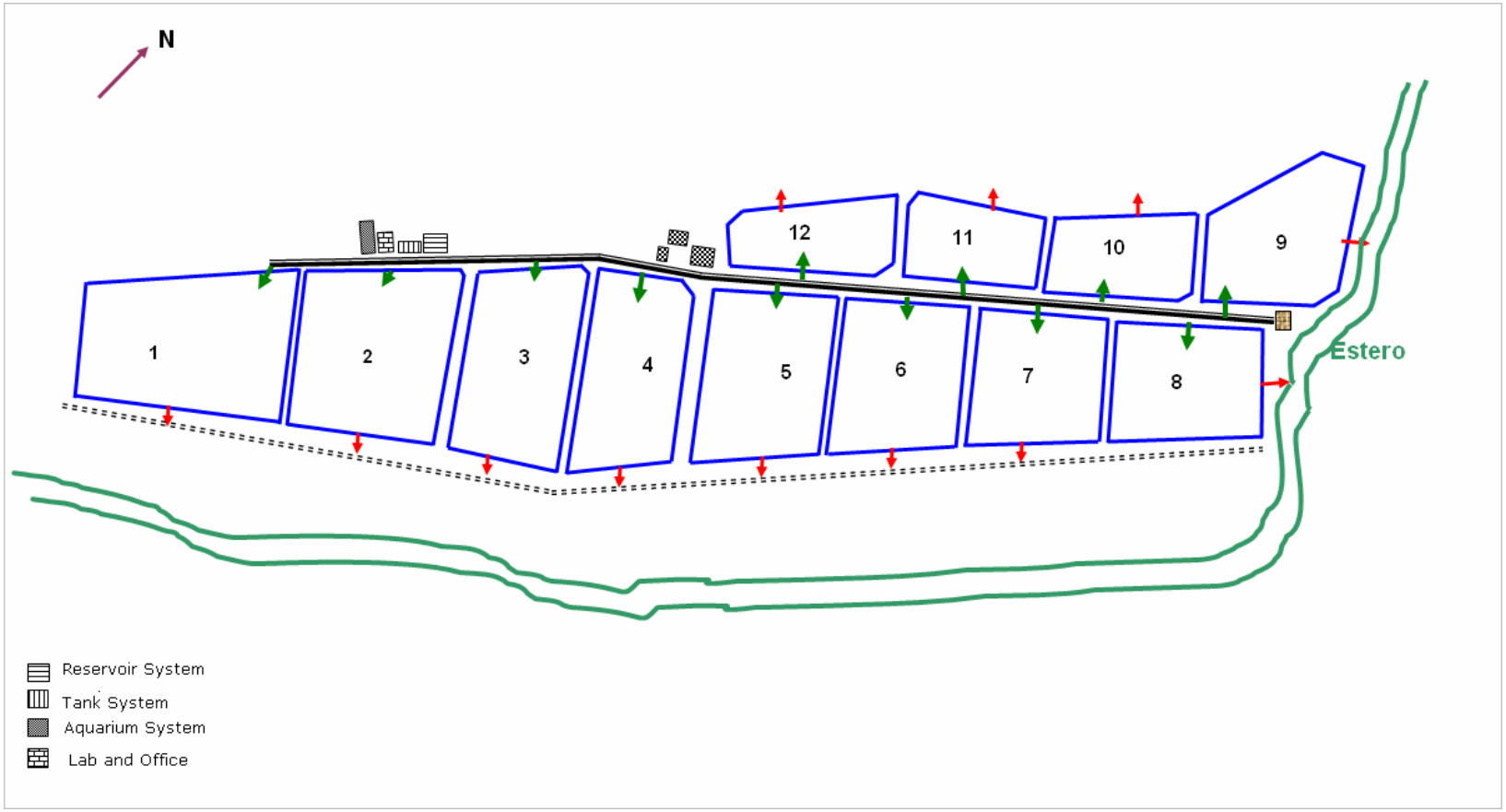

Figure 2.1 Diagram of the semi-closed intensive shrimp farm (LATIMAR) and the Aquatic Experimental Station (AES) in Tumbes, Peru. Image is drawn approximately to scale.

Characterization of phytoplankton in the reservoir tank

The experiment in the reservoir tank occurred from 15 March to 9 May 2008 (total of 56 d). Water initially pumped from the estuary "El Venado", was used to fill the reservoir tank to a depth of 100 cm (28,270 L). The following day, hydroxyl chloride was added yielding a chlorine level of 4 ppm. Water was then aerated for 24 h to allow for evaporation of chlorine. Concentrations of nitrogen and phosphorus were determined the first day of evaluation (three days post-filling) using a YSI 9000® photometer. Water was then fertilized to yield a N/P and Si/N ratio of 15 and 1.25, respectively. Fertilization with inorganic nutrients occurred at noon using Nutrilake® (as above) and sodium silicate Nutricil® (23% silicate). Also, 30 g of ground 35% crude protein (Nicovita®, Alicorp, Lima, Peru) commercial feed (5.6% nitrogen, 0.85% phosphorus wt:wt) and 30 g of commercial organic fertilizer (Nicovita®, Alicorp, Lima, Peru: 2.1% nitrogen and 0.65% phosphorus wt:wt) were added. The commercial organic fertilizer consisted of a mixture of wheat middlings and defatted soybean meal. It was also added daily, and served as a source of carbon, nitrogen and phosphorus in order to maintain continuous organic nutrients in the water for the algal community. After seven days, addition of Nutrilake® and triple super phosphate was suspended. Nutricil® was continuously added at a rate of 140 g d^{-1} (the equivalent of 50 kg ha^{-1} d^{-1}) and ground commercial diet and organic fertilizer were maintained at the same 30 g per day rate for the entire experimental period in order to stimulate the growth of diatoms and avoid predominance of cyanobacteria. At day 21 and 46 (8 April 2008 and 3 May 2008), 50% of the reservoir water (14,000 L) was exchanged with non-chlorinated estuarine water.

During water exchange, levels of nitrogen and phosphorus were determined in order to maintain the ratios of N/P and Si/N at 15 and 1.25, respectively. Again, after these two water exchanges and after seven days of fertilization, Nutrilake® and triple super phosphate were suspended and Nutricil® was added at a rate of 140 g d^{-1} (the equivalent of 50 kg ha^{-1} d^{-1}). Ground commercial feed and organic fertilizer were also each added at a rate of 30 g per day. Water quality factors were monitored in a similar manner as reported above. In addition, Secchi disc depth (Walker et al. 2007) and luminosity (Milwaukee® SM 700 Lx light meter) were determined daily at noon.

Phytoplankton identification, cell counts and chlorophyll analysis were also determined daily. A 1-L water sample was collected at noon at a depth of 30 cm from the reservoir tank, and preserved by addition of Lugol's solution (Throndsen 1978). After allowing for settling, taxonomic and cell count analysis were conducted. Microalgae counts were undertaken as described above. Analysis of chlorophylls *a*, *b* and *c* was by the method of Strickland & Parsons (1972) using the spectrophotometric equations from Jeffrey & Humphrey (1975).

Results

Characterization of phytoplankton in ponds

Water temperature (C), DO (mg L^{-1}), pH and salinity (g L^{-1}) during the months sampled were statistically different ($P<0.05$) among the 12 ponds (Table 2.1). However, NO_2-N (mg L^{-1}) was not statically different among the 12 ponds. Water temperature, DO and pH were statistically ($P<0.05$) higher during the afternoon readings. DO levels ranged in the morning from 0.6 mg L^{-1} in pond 12 to 8.1 mg L^{-1} in pond ten and in the afternoon from

Table 2.1 Water quality parameters of study ponds. Numbers in parenthesis are SD of the means. n= 378 for temperature, DO and pH and 17 for nitrite and salinity

Pond	Temperature (°C)			Dissolved oxygen (mg L^{-1})			pH			NO_2-N (mg L^{-1})			Salinity (g L^{-1})		
	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
1 AM	30.6 (1.1)	26.0	36.8	2.8 (0.72)	1.4	4.9	7.5 (0.34)	7.0	8.6	1.2 (1.89)	0.1	7.0	30 (3.0)	24	35
PM	31.9 (1.1)	27.4	36.4	3.5 (1.10)	1.7	8.6	7.8 (0.60)	3.0	8.9						
2 AM	31.2 (1.3)	25.4	38.7	2.5 (0.70)	1.4	4.9	7.4 (0.48)	2.2	8.8	4.5 (5.35)	0.0	20.3	29 (3.8)	22	36
PM	32.3 (1.2)	26.9	34.2	2.9 (0.89)	1.5	5.9	7.6 (0.59)	2.6	8.6						
3 AM	30.9 (1.1)	27.3	32.7	2.7 (0.89)	1.4	7.9	7.4 (0.34)	3.6	8.0	1.3 (2.45)	0.0	7.8	28 (2.2)	24	32
PM	32.1 (1.3)	28.0	38.9	3.1 (0.89)	1.7	7.2	7.7 (0.22)	7.2	8.6						
4 AM	30.9 (1.2)	27.0	32.9	2.8 (0.87)	1.5	7.7	7.5 (0.16)	7.1	8.1	0.8 (1.36)	0.0	5.0	29 (3.0)	20	33
PM	32.0 (1.5)	28.0	34.7	3.2 (0.87)	1.6	5.9	7.8 (0.38)	2.9	8.4						
5 AM	30.8 (2.3)	26.8	34.3	2.6 (0.64)	1.4	4.2	7.4 (0.53)	2.3	8.5	1.2 (2.12)	0.0	8.0	32 (2.9)	25	36
PM	32.1 (1.1)	28.2	33.9	3.3 (0.85)	1.6	5.2	7.7 (0.65)	2.3	8.7						
6 AM	31.3 (1.2)	26.5	33.7	2.5 (0.76)	1.5	7.7	7.5 (0.30)	6.9	8.7	1.7 (2.32)	0.0	8.3	31 (2.4)	28	36
PM	32.4 (1.3)	27.4	34.3	2.9 (0.80)	1.6	5.4	7.7 (0.52)	2.3	8.9						

Table 2.1 Continued

Pond	Temperature (°C)			Dissolved oxygen (mg L^{-1})			pH			NO_2-N (mg L^{-1})			Salinity (g L^{-1})		
	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.
7 AM	31.1 (1.0)	26.2	32.7	2.8 (0.98)	1.4	7.8	7.6 (0.20)	7.2	8.3	1.6 (3.07)	0.0	10.8	29 (1.4)	26	31
PM	32.2 (1.1)	27.2	37.8	3.2 (0.89)	1.2	6.2	7.8 (0.44)	2.6	8.5						
8 AM	31.6 (0.7)	28.4	32.8	2.8 (0.89)	1.3	7.7	7.6 (0.24)	7.1	8.5	3.1 (4.13)	0.0	12.9	27 (3.5)	23	37
PM	33.1 (3.7)	29.3	34.0	3.2 (1.03)	1.5	7.5	7.9 (0.29)	7.4	8.9						
9 AM	31.5 (0.8)	28.5	34.5	2.7 (1.09)	1.4	7.5	7.6 (0.52)	2.1	8.7	3.3 (4.75)	0.0	12.5	28 (4.3)	23	36
PM	32.7 (0.8)	29.8	34.4	3.2 (1.15)	1.6	7.3	7.8 (0.33)	6.3	9.1						
10 AM	31.4 (0.8)	28.2	33.1	2.6 (0.78)	1.2	8.1	7.6 (0.28)	7.9	8.6	2.7 (4.02)	0.0	14.2	25 (2.3)	22	32
PM	32.5 (1.1)	24.2	36.4	3.2 (0.94)	1.2	7.4	7.9 (0.51)	3.6	9.9						
11 AM	31.6 (1.1)	27.0	33.6	2.5 (0.71)	1.1	4.9	7.5 (0.56)	2.4	8.7	2.1 (2.50)	0.1	10.0	29 (4.3)	22	35
PM	32.7 (1.0)	28.4	34.6	3.0 (0.92)	1.3	5.9	7.8 (0.65)	2.2	9.9						
12 AM	30.6 (1.6)	21.9	32.9	2.3 (0.85)	0.6	6.8	7.6 (0.33)	7.1	8.6	1.8 (3.73)	0.0	14.2	29 (4.3)	21	36
PM	31.9 (1.5)	26.2	37.9	3.0 (0.96)	1.6	7.6	7.8 (0.33)	7.0	8.8						

1.2 mg L^{-1} in pond seven and 8.6 to 10 mg L^{-1} in pond one. The Cyanophyta appeared to be the predominant algal division followed by Chrysophyta, Pyrrophytes, Dynophytes and Euglenophyta (Table 2.2). A total of 23 genera, representing four divisions of algae, were identified from the ponds at LATIMAR throughout the study period. Overall, the most commonly observed genera were *Oscillatoria* and *Crococcus*, with *Melosira, Navicula, Nitzchia*, *Thallasiosira,* and *Fragilaria* being the most common genera of diatoms.

On the first sampling date (11 November 2006), the phytoplankton community was dominated by cyanobacteria which comprised 75 - 85 % of the total cells counted (Fig. 2.2). However, on 14 November, the dominant species changed to Chrysophyta (65% of total cell counts). In this case, the proportion of cyanobacteria declined to 25%. From 17 November to the end of the sampling dates (28 July 2007) the cyanobacteria were dominant (70 - 95%).

Mean microalgae cell counts from November 2006 to June 2007, ranged from 168,650 cells mL^{-1} in Pond 7 to 313,900 cells mL^{-1} in pond 4 (Fig. 2.3). The highest concentration of cyanobacteria was observed in pond five with a mean of 160,727 c mL^{-1} (91%). Pond ten had the highest mean cell density of diatoms (95,636 c mL^{-1}; 38% of total).

Characterization of the phytoplankton in the reservoir tank

The water quality conditions of the AEC reservoir tank during the experimental period are shown in Table 2.3. No significant differences (P>0.05) in water temperature, DO, pH and salinity were observed during the 51 days of sampling. Dissolved oxygen, water

Table 2.2 Percent count (cell mL^{-1}) of each microalgae genera identified in study ponds from November 2006 to July 2007

Division	Genus	Frequency %	Division	Genus	Frequency %
Chrysophyta	*Melosira*	3.9	Cyanobacteria	*Oscillatoria*	54.1
	Navicula	3.6		*Crococcus*	21.6
	Nitzchia	3.8		*Anabaena*	0.5
	Thallasiosira	2.8		*Spirulina*	0.1
	Fragilaria	1.3	% Total Division		76.3
	Chaetoceros	0.8			
	Cymbella	0.5	Pyrrophyte	*Phacus*	100
	Pleurosigma	0.4	% Total Division		5.1
	Amphipora	0.2			
	Coscinodiscus	0.1	Euglenophyte	*Euglena sp*	100
	Coconeis	0.1	% Total Division		1.0
	Bidulfia	0.1			
	Risolenia	0.05			
	Gyrosigma	0.04			
	Scheletonema	0.04			
	Pinnularia	0.02			
	Bacteriosira	0.01			
% Total Division		17.60			

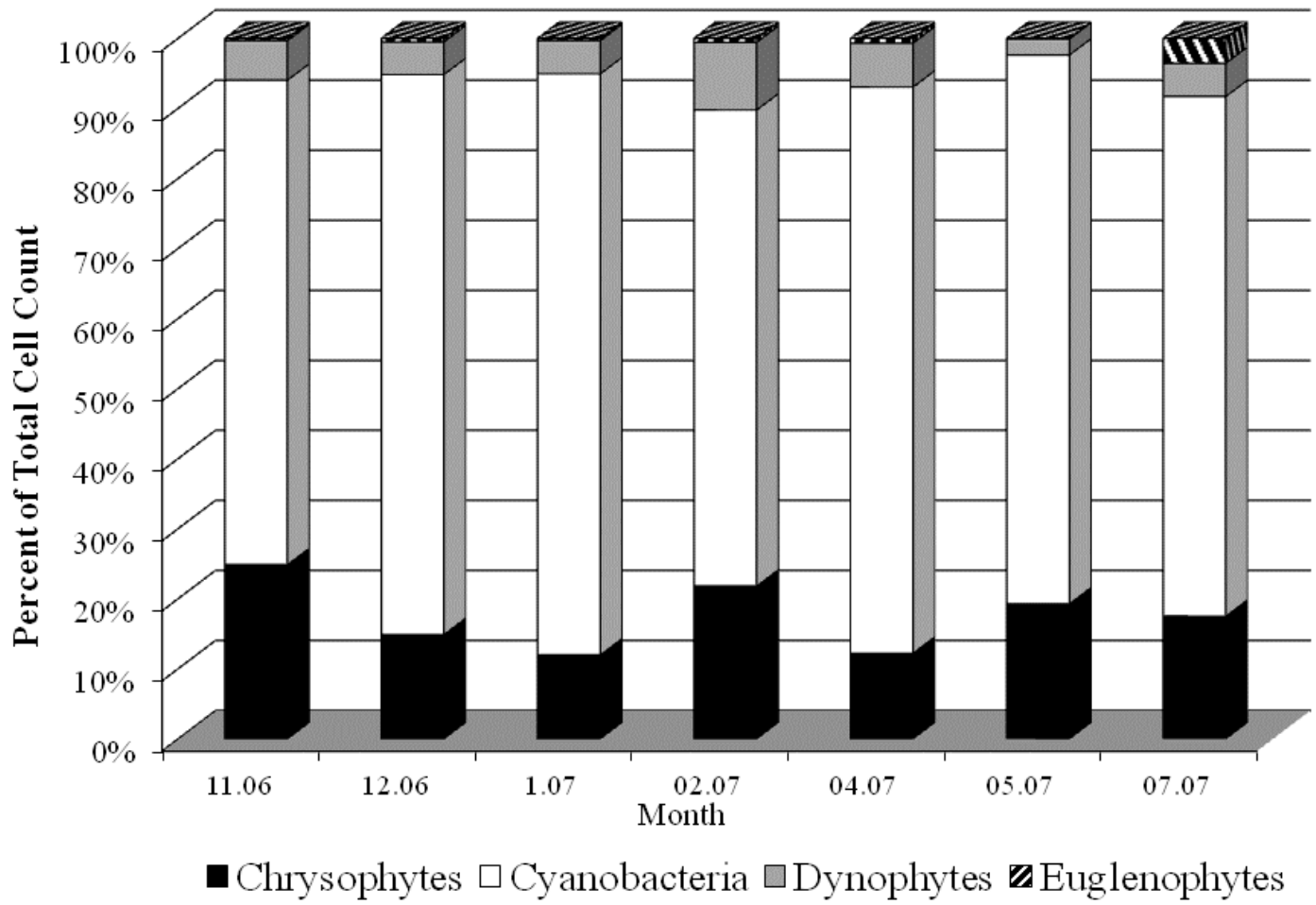

Figure 2.2 Mean phytoplankton community structure by month from November 2006 to July 2007.

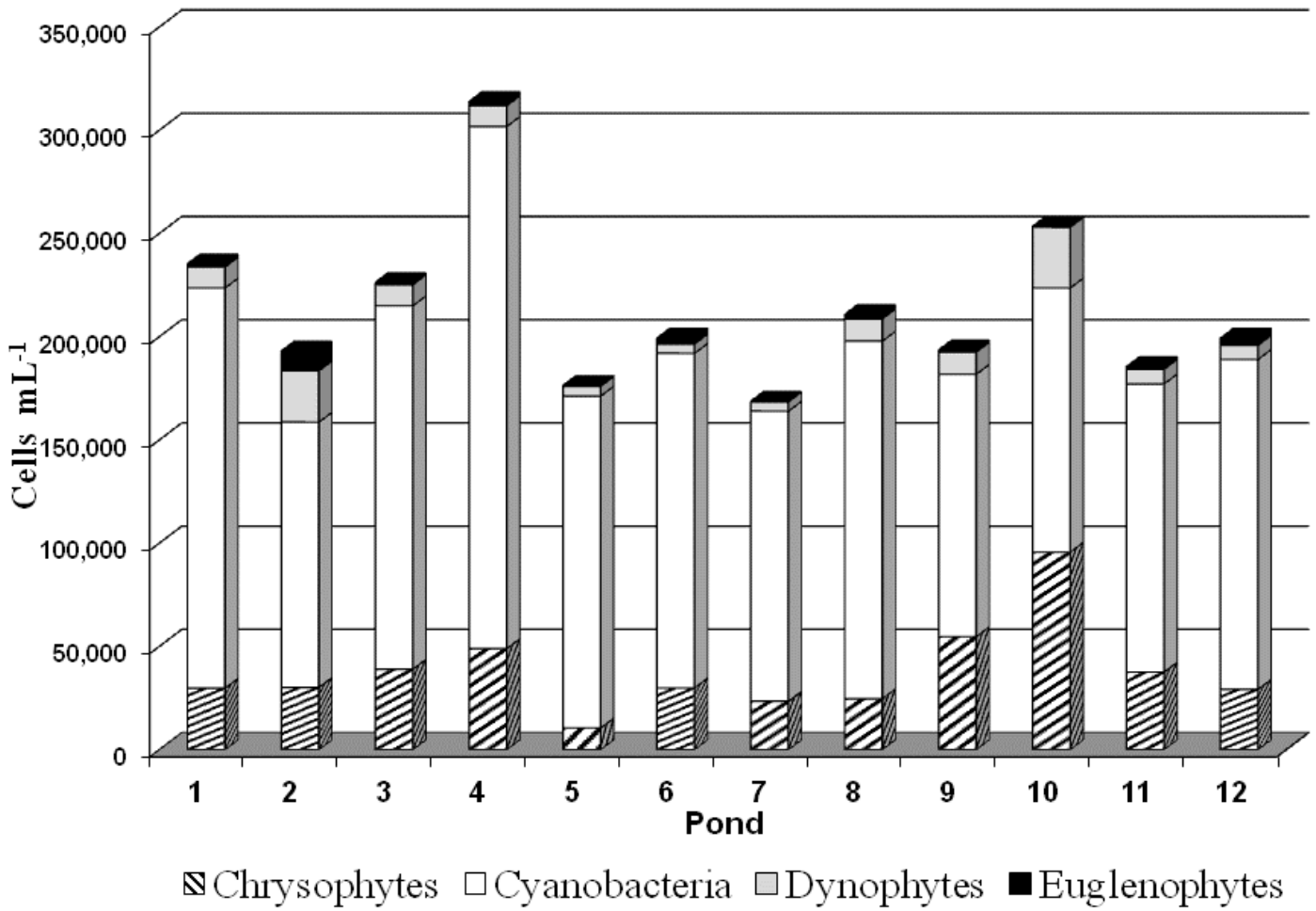

Figure 2.3 Phytoplankton by cell count of 12 semi-closed intensive shrimp culture ponds in Tumbes – Peru between November 2006 and June 2007.

temperature and pH were higher during the afternoon readings, but did not vary substantially at each sampling time (AM and PM; Table 2.3). Secchi depth and levels of chlorophyll *a*, *b* and *c* were relatively low during the 52-days of trial, especially during the first 35 days. Mean luminosity was 10,487 ± 2,972 lx cm^{-2}, with a minimum of 4,930 lx cm^{-2} and a maximum of 22,900 lx cm^{-2} depending on the daylight and amount of shadow from the clouds.

Water from the "El Venado" estuary, used to initially fill the reservoir tank, contained diatoms (75%), cyanobacteria (20%) and chlorophytes (5%) and were ultimately eliminated as a result of disinfection with chlorine. The water used to exchange 50% of the reservoir water on 8 April 2008 consisted of cyanophytes (65%), chlorophytes (15%), diatoms (10%) and pyrrophytes (10%; Fig. 2.4). In contrast, estuary water used on 3 May 2008 consisted of 87.5% cyanophytes and 12.5% chlorophytes.

The microalgae cell count from 19 March to 9 May 2008, varied from 1,150 c mL^{-1} on day one to 12,595 c mL^{-1} on day eight (Fig. 2.5). Diatoms were the dominant species of microalgae during the first 14 d of the study; whereas, cyanobacteria dominated during d 17 to 35 and the last five days of the trial, with cell counts ranging from 650 to 2,470 c mL^{-1}. Diatoms, cyanophytes and chlorophytes began to increase ten days after the first water exchange (day 36 of the trial). Secchi depth decreased to approximately 30 cm as concentrations of microalgae increased from 160,000 to 265,500 c mL^{-1}.

Table 2.3 Variation in environmental criteria from 19 March to 9 May 2008 (52 d) in the reservoir tank. Numbers in parenthesis are SD of the mean. A total of 104 observations were made for temperature, DO and pH; and 52 for Secchi depth, luminosity and chlorophyll

Factor	AM	Min	Max	PM	Min	Max
Temperature (°C)	31.0 (0.9)	28.1	32.8	32.5 (1.0)	30.1	34.6
DO (mg L^{-1})	5.5 (0.9)	4.1	9.8	8.1 (1.6)	5.6	13.2
pH	8.6 (0.3)	7.5	9.2	8.8 (0.3)	8.1	9.3
Salinity (g L^{-1})				28.3 (0.9)	26.3	29.8
Secchi Depth (cm)				62 (24)	24	100
Luminosity (Lx cm^{-2})				10487 (2,972)	4,930	22,900
Chl - *a* (mg m^{-3})				20.1 (15.3)	1.1	61.7
Chl - *b* (mg m^{-3})				1.03 (1.7)	1.6	6.3
Chl - *c* (mg m^{-3})				4.7 (3.8)	0.4	13.8.

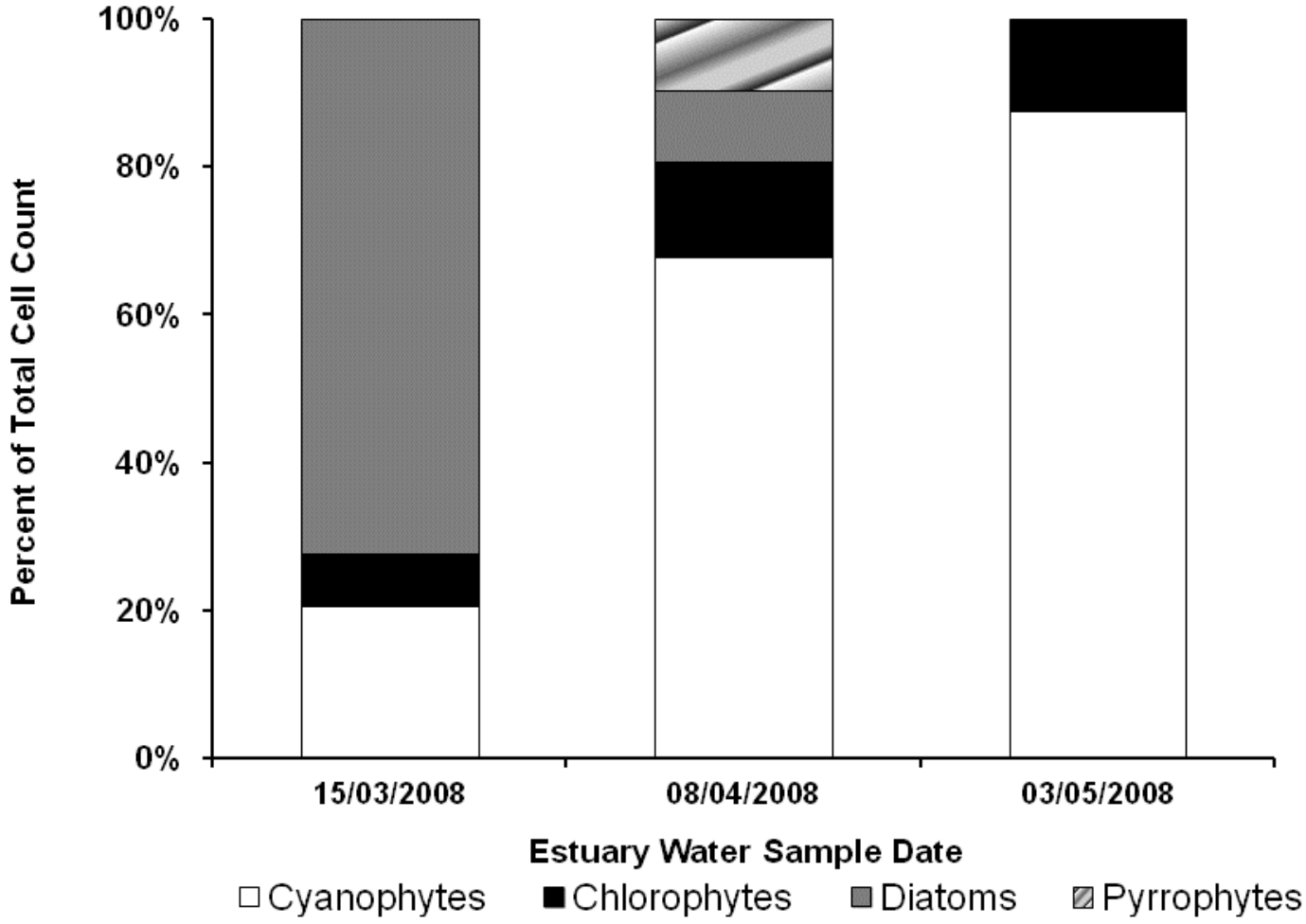

Figure 2.4 Phytoplankton community structure, by division, of estuary water used to exchange the reservoir tank.

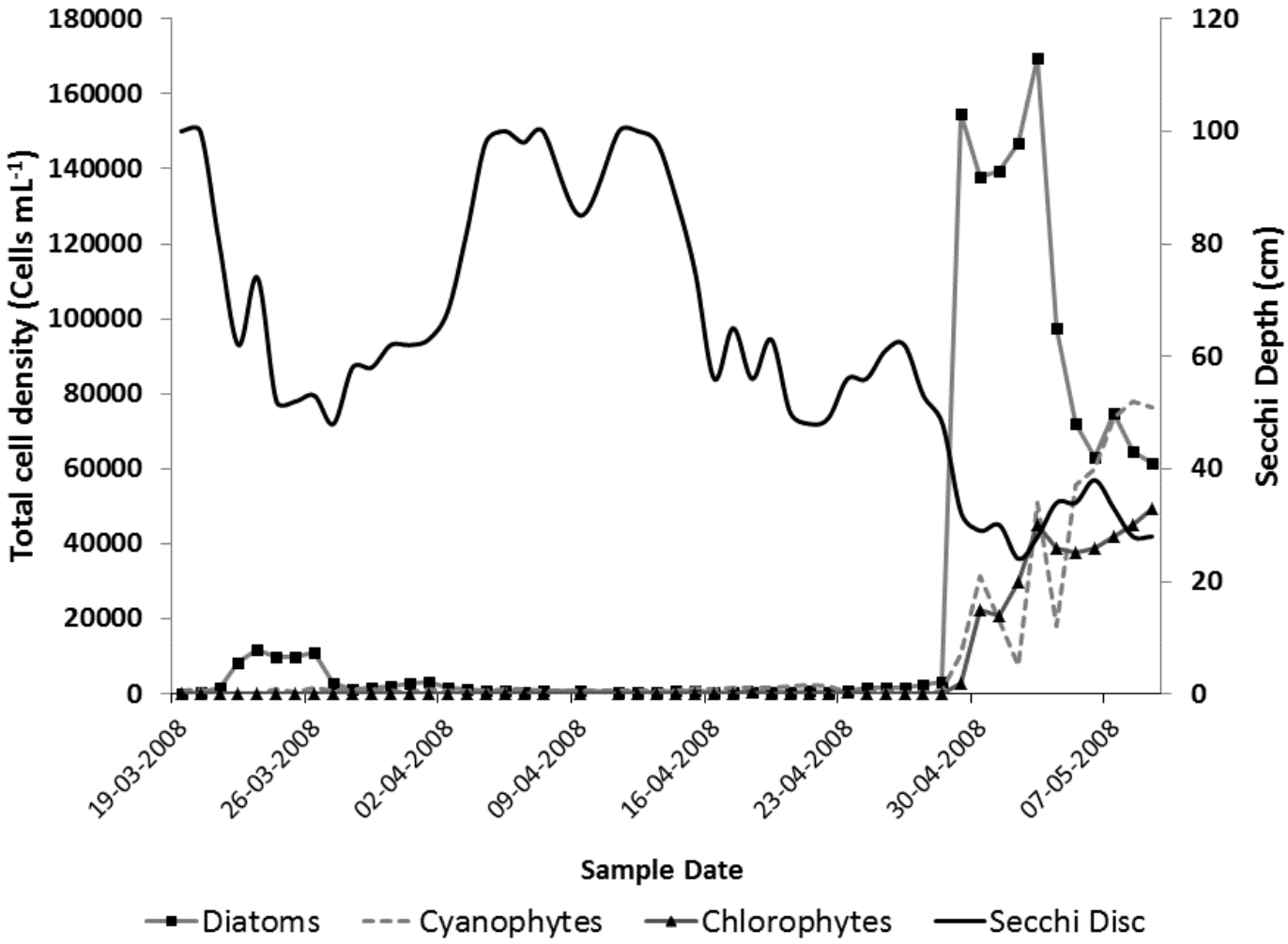

Figure 2.5 Phytoplankton cell count vs. Secchi depth in reservoir tank from 19 March to 09 May 2008 (52 d).

On the first sampling date (19 March 2008), the phytoplankton community was dominated by cyanobacteria which comprised 82% of total cells counted (Fig. 2.6). From day three to nine, diatoms comprised more than 80% of the total cell count, compared to cyanophytes. From day ten (28 March 2008) to the end of sampling (7 April 2008), cyanophytes decreased from 50 to 12% on day 13, and as high as 60% until day 35 (22 April 2008) when diatoms began to dominate and rapidly increase in number. This continued until day 42 (29 April 2008) at which point they comprised 99% of total cells. At day 42, cyanophytes and chlorophytes began to increase in number, comprising 40% and 26% of total cells, respectively. This continued until day 52, when the trial ended. During this same time period, diatoms comprised 32% of the total cell count.

Diatoms (e.g., *Chaetoceros* sp., *Nitzchia* sp., *Navicula* sp., *Gyrosigma* sp.) comprised 61% of the total cells, followed by cyanophytes (e.g., *Oscillatoria* sp.) at 23% and chlorophytes (e.g., *Chlamydomonas* sp.) at 16%. Pyrrophytes were present in very small concentrations and euglenophytes were apparently completely absent. A total of 13 genera, representing three algal divisions, were identified from the reservoir tank during the 53 days of sampling (Table 2.4).

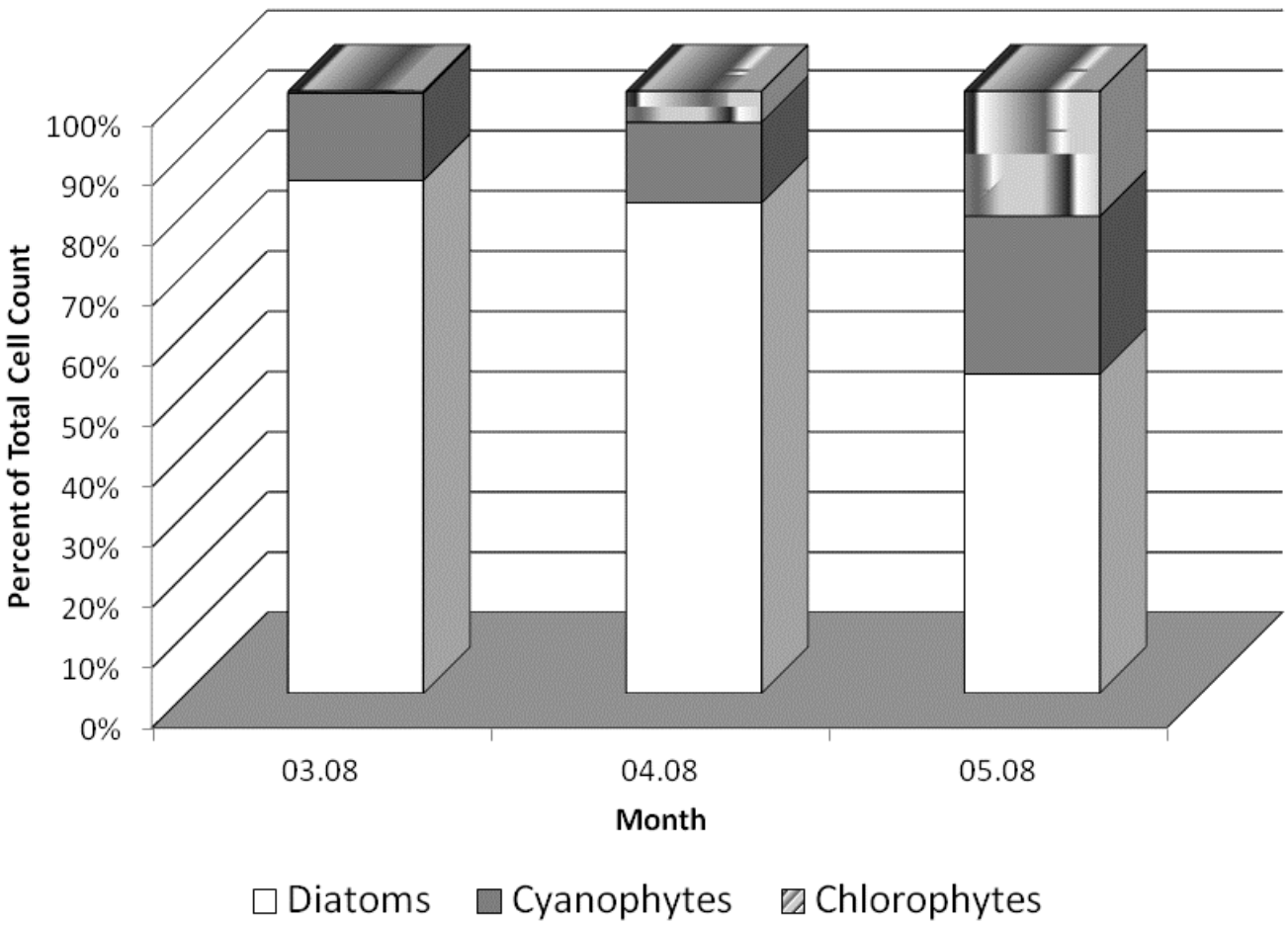

Figure 2.6 Phytoplankton community structure, by division, as percentage of total sample for reservoir tank. Samples taken from 19 March to 9 May 2008 (52 d).

Table 2.4 Percent count (cell ml^{-1}) of each microalgae genera identified in reservoir tank from 19 March to 9 May 2008

Division	Genus	Frequency %	Division	Genus	Frequency %
Diatoms	*Chaetoceros*	52.7	Cyanobacteria	*Oscillatoria*	22.6
	Nitzchia	2.7		*Chroococcus*	0.1
	Navicula	2.2		*Gleocapsa*	0.1
	Gyrosigma	2.1		*Spirulina*	0.03
	Cymbella	0.8	% Total Division		22.8
	Amphipleura	0.2			
	Fragilaria	0.03	Chlorophytes	*Chlamydomonas*	16.4
% Total Division		60.7		*Oocysts*	0.07
			% Total Division		16.5

The predominant species of chlorophyll was chl-*a* (20.1 mg m^{-3}) followed by chl-*c* (4.7 mg m^{-3} - Table 2.3). Total chlorophyll followed the pattern of cell count and Secchi depth independent of the predominant species of microalgae (Fig. 2.7). Transparency varied from 100 to 24 cm and total chlorophyll from 1.9 to 77.9 mg m^{-3}. As level of transparency decreased, total chlorophyll increased. Maximum chlorophyll (77.2 mg m^{-3}) was observed when transparency reached 28 cm on May 3; whereas, minimum chlorophyll (1.9 mg m^{-3}) was observed when Secchi depth reached 85 cm on April 9 simultaneous with water exchange.

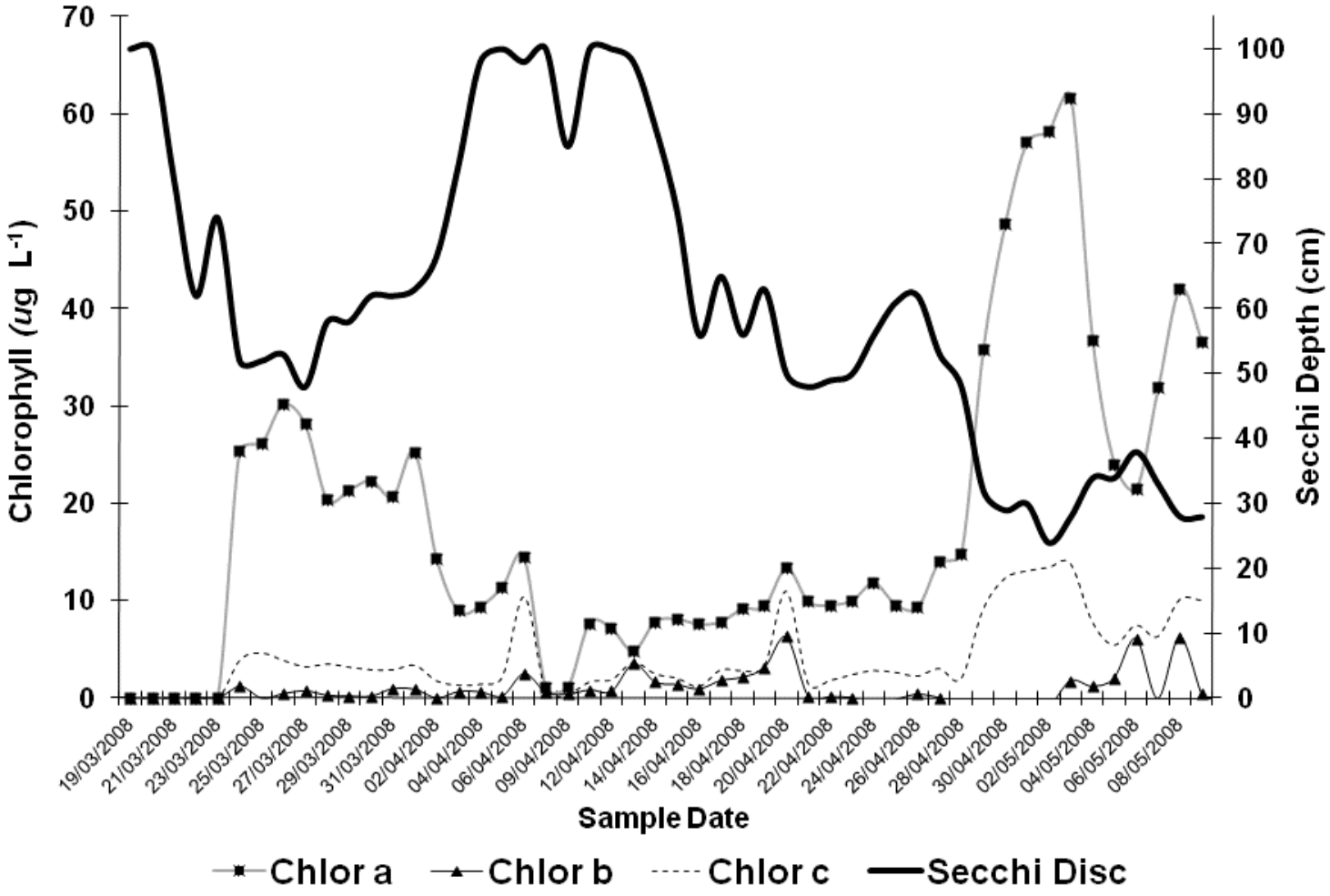

Figure 2.7 Chlorophyll and Secchi depth levels in reservoir tank from 19 March to 9 May 2008 (52 d.).

Discussion

Phytoplankton community structure in ponds

The phytoplankton community structure observed during the study period was typical of shrimp culture ponds. Phytoplankton communities in coastal shrimp ponds are often dominated by cyanobacteria and/or chrysophytes (Boyd 1989; Cortez-Altamirano, Paez-Osuna, Guerrero-Galvan & Esparza-Leal 1995; Tookwinas & Songsangjinda 1999; Yusoff, Zubaidah, Matias & Kwan 2002; Alonso-Rodriguez & Paez-Osuna 2003; McIntosh *et al.* 2006). On the other hand, Burford (1997) suggested that shrimp ponds can display higher cell densities of chlorophytes and cyanobacteria than chrysophytes in relation to the water source. These differences are generally attributed to decreased light availability, higher nutrient levels and decreased silica content (McIntosh *et al*. 2006).

The dominance of cyanobacteria for most of the study period in all 12 shrimp culture ponds on the LATIMAR farm was probably due to the high levels of phosphorus in the system and nitrogen/phosphorus imbalance, both mainly caused by high amounts of feed inclusion (35% CP diet contains 5.6% N and 0.74% P which results in a N/P ratio of 7.6). Researchers have reported that the major source of nutrients in pond water is feed (Boyd 1989; Wang 1990; Briggs & Funge-Smith 1994; Tookwinas & Songsangjinda 1999; Alonso-Rodriguez & Paez-Osuna 2003). Shrimp feed is rich in nitrogen and phosphorus, containing 6.1 – 7.4% N and 1 – 1.5% P (Chiu 1988). The factors considered important in development of cyanobacteria blooms include high concentration of ammonium nitrogen (Blomqvist, Petterson & Hyenstrand 1994; Ferber, Levine, Lini & Livingston 2004; Marino, Chan, Howarth, Pace & Likens 2006), high

levels of phosphorus (Schindler 1977; Trimbee & Prepas 1978; Watson, McCauley & Downing 1997), low total nitrogen/total phosphorus ratios (Smith 1983; Lee, Jang, Kim, Yoon & Oh 2000; Moisander, Steppe, Hall, Kuparinen & Paerl 2003; Alonso-Rodriguez & Paez-Osuna 2003; Wacklin 2006; Watzin, Fuller, Kreider, Couture & Levine 2006), high pH and low CO_2 availability (Reynolds 1973) and physical factors such as high temperature (Robarts & Zohary 1987), turbulence and mixing (Steinberg & Hartmann 1988; Oliver & Ganf 2000; Paerl, Fulton, Moisander & Dyble 2001; Moisander, Hench, Kononen & Paerl 2002), and high light intensity (Paerl, Bland, Bowles & Haibach 1985; Tandeau de Marsac & Houmard 1993; Scheffer 1998; Vörös, Callieri, Balogh & Bertoni 1998; Postius & Ernst 1999; Mur, Skulberg & Utkilen 1999; Ferris & Palenik, 1998; Oliver & Ganf 2000; Callieri & Stockner 2002; Stomp, Huisman, De Jongh, Veraart, Gerla, Rijkeboer, Ibelings, Wollenzien & Stal 2004).

Zimba, Grimm & Dionigi (2001) reported that freshwater catfish earthen pond systems were dominated by cyanobacteria, which can lead to taste and odor problems in the fish. These ponds typically receive large nutrient inputs from excess feed, fish excreta and sediment mineralization/resuspension. This is the result of the large surface area:volume of these shallow water systems (depth < 1.5 m) as well as 30% conversion of feed into fish biomass (Tucker & Boyd 1985). Two of the predominant species found in these freshwater catfish ponds, *Anabaena* sp. and *Oscillatoria* sp. (50% of samples) and *Aphanizomenon* sp. (20% of samples), were less common in the present study (Table 2.4), probably due to the higher level of salinity of the estuary water.

The diatoms *Nitzchia* sp. followed by *Amphora* sp., *Navicula* sp. and *Pleurosigma* sp. were the most common genera of microalgae found during a three-cycle study in a Caribbean shrimp farm in Venezuela. There, shrimp were stocked at 15 and 20 m^{-2} (Velasquez *et al*. 2007). The dominant species of microalgae in that study could have differed from the present due to improved water quality and availability of silicates in sediments, as well as general water column characteristic of these semi-intensive culture systems found in the Caribbean Sea area. However, high numbers of cyanobacteria were observed in the third cycle, due to eutrophication of the ponds. As previously mentioned, cyanobacteria become enriched with phosphorus and reproduce rapidly as the amount of feed increases. Diatoms require specific levels of silicate, which was not applied as a supplement at LATIMAR ponds. Hence, this type of microalgae did not dominate at any time during the sampling period.

Microalgae community structure was determined during a five-month period in 20 low-water-exchange shrimp culture ponds in Eastern Thailand (Tookwinas & Songsangjinda 1999). Densities varied from 33 to 110 shrimp m^{-2}. During the second, third and fourth months, the cyanobacterium *Trichodesmium* sp. was dominant (24.1, 62.8 and 30.9%, respectively). During the first month *Nitzchia* sp. (18.7%) and *Lyngbya* sp. (9.9%) dominated; whereas, the chlorophyte, *Chlorococcus* sp. comprised 28.8% of the assemblage during the fifth month. The dominance of cyanobacteria in both Thai and LATIMAR could be principally due to similarities in stocking density, type of Pacific estuarine water and reduced water exchange typically used to minimize stress on shrimp.

Phytoplankton community in the reservoir tank

Water used during the first stage of the present study was phytoplankton free, due to disinfection and microalgae air spores were likely the main source resulting in colonization of pond water. The limited amount of surface water in the reservoir which was greenhouse enclosed did not allow enough open space for algae colonization and propagation. When non-chlorinated estuarine water was eventually exchanged (d 22), diatoms proliferated, even though incoming water consisted primarily of cyanobacteria (65%) and diatoms (10%). The positive relationship between nutrient loading and productivity in freshwater and marine environments has been well-established (Schindler 1977; Schindler 1978; Smith 1982; Pick & Lean 1987; Hecky & Kilham 1988; Boyd & Daniels 1993; USEPA 2000; Moisander *et al*. 2003; Walker, Younos & Zipper 2007). Thus, diatom proliferation in the reservoir tank was probably due to the N/P ratio used and the silicate applied during the fertilization program. According to Smith (1983), a dramatic tendency for blue-green algal blooms occurs when epilimnetic N/P ratios fall below 29 by weight, and for blue-green algae to be rare when the N/P ratio exceeds this value. Tilman, Kilham & Kilham (1982) suggested that blue-green algae (both those that fix nitrogen and those that do not) are generally inferior to diatoms as phosphorus competitors, indicating that blue-green algae should typically be dominant in lakes with low N/P ratios (in which most phytoplankton species would be nitrogen limited) and rare in lakes with high N/P ratios. Flett, Schlinder, Hamilton & Campbell (1980), found that nitrogen-fixing blue-green algae were typically associated with lakes having N/P supply

ratios less than 10 by weight and were mostly absent from those with a greater N/P supply ratio.

Watson *et al.* (1997) found that despite diatoms having a smaller range in size and shape than cyanobacteria, they contributed a comparable percentage to total biomass in 91 northern temperate lakes. Diatoms are well adapted to a broad range of nutrient regimes (Willen 1991; Corbelas & Rojo 1994). Efficient nutrient uptake, especially silicate, may favor pinnate diatoms in oligotrophic environments (Sterner 1990; Velasquez *et al.* 2007), and centric diatoms, with higher growth rates and lower sinking losses, in eutrophic lakes, particularly in mixed systems (Corbelas & Rojo 1994). On the other hand, cyanobacteria have been shown to increase their proportion of total biomass at concentrations as low as 1.4 ppb total phosphorus (Watson *et al*. 1997). These findings were corroborated during subsequent days of the present study, after water was exchanged the second time (d 42). At this point, diatoms were less prevalent in the total cell count; whereas, cyanobateria and chlorophytes continued to increase until the end of the study. Because of this pattern, it might be inferred that an additional water exchange and re-fertilization, with a N/P ratio of 15 and adequate silicate levels could result in a phytoplankton assemblage containing a higher proportion of diatoms. This assumption should be confirmed under normal pond operation conditions.

It is clear that management decisions on nutrient control measures must be based on controlled field tests as well as simple laboratory bioassays (Schindler 1977). For example, Zimba *et al.* (2001) found that pond size played a significant role in phytoplankton abundance. Thus, differences found in microalgae community structure

among the 12 LATIMAR ponds and the reservoir tank system at the AES, should be considered jointly. Microalgae populations in the reservoir were different from those in the ponds, probably due to internal and external variables such as a smaller controlled environment, different nutrient input (e.g., silicate, phosphorus and nitrogen) in the fertilization regime, amount of water used, light intensity and the lack of shrimp, grazers and predators. The assemblage of species comprising the microalgae community is subject to many variables (e.g., temperature, salinity, DO, pH, light intensity and nutrient balance). A good fertilization program could help to enhance presence of a desirable species of microalgae while simultaneously reducing unwanted ones. Despite known relationships with these controlling factors, substantial environmental variation (i.e., rate of change) could affect unpredictable change.

Blooms of cyanobacteria usually occur during warm periods, at temperatures above 20 °C (Robarts & Zohary 1987). Both field and laboratory experiments (Reynolds 1984; Robarts & Zohary 1987) have supported the hypothesis that elevated temperatures favor presence of cyanobacteria over that of other phytoplankton (Tilman *et al.* 1982; McQueen & Lean 1987). Cyanobacteria generally have higher temperature optima (>25 °C) for growth, photosynthesis and respiration than do green algae and diatoms, although direct temperature effects probably act synergistically with other factors in the process (Robarts & Zohary 1987). Diatoms dominate a broader range of nutrient conditions (Si/P and N/P) below 14 °C; whereas, chlorophytes and cyanobacteria, specially *Oscillatoria* sp., dominate a wider range of nutrient ratios at temperatures of 17 °C and 24 °C (Tilman *et al*. 1982). Trimbee & Harris (1984) stated that seasonal

succession also may affect both chl-*a* levels and community structure. Thus, although it is likely that the assemblage of microalgae could be managed by an appropriate program of fertilization in the reservoir tanks, the changing of shapes and sizes of containers, with different water parameters, as well as including shrimp and feed in the experimental tanks and ponds, could result in other dynamics. This could make it more difficult to stabilize beneficial microalgae communities. For this reason, it is important to cautiously apply results from a smaller controlled system to a larger pond.

A number of methods are available for quantification of microalgae, including cell counts, biovolume, and algal pigments (e.g., chlorophylls, carotenoids and phycobilins). The biomass of phytoplankton in lakes and reservoirs is often estimated only by measuring the amount of chl-*a*, the predominant green pigment used in photosynthesis (Walker *et al.* 2007). Some authors suggest recording total chlorophyll pigments because all current methods of measuring chlorophyll-*a* concentration ignore some interference (Carlson & Simpson 1996). In the present study, the determination that levels of chlorophyll-*a*, -*b* and -*c* varied according to the type of predominant microalgae, suggests that cell counts and chlorophyll levels could be used as a balanced and complementary approach to monitor microalgae assemblages in the field. Zimba *et al*. (2001) corroborated that cell count data were generally strongly related to chl-*a* concentration in catfish ponds and that higher pigment values were associated with larger pond sizes. Also, chl-*a* level in inland low-salinity shrimp ponds, increased over the course of a 5-month growing season, with the level being higher in small ponds compared to larger ponds (McIntosh *et al*. 2006). Furthermore, USEPA (2004) used a

comprehensive baseline of 11 years of chl-*a* values as an indicator of amount of phytoplankton suspended in the water column. They determined that increases in chl-a values were not detected, and thus the bay they studied (e.g., Hervey Bay, Australia) maintained a stable trophic status and did not present any increasing trend or risk of eutrophication. Morabito, Ruggio & Panzani (2001) also found a strong correlation between phytoplankton total bio-volume and chl-*a* concentrations over a 13-yr period in Lake Orta, Italy.

Analysis of costs and time consumed should be taken into consideration when evaluating a method to determine natural productivity in ponds. Associations between chlorophyll levels and nutrients are not currently available for shrimp ponds; however, it is expected that a positive correlation exists (McIntosh *et al.* 2006). Factors such as fertilization rates, day length and other physiochemical variables and many others are directly related to both chl-*a* level and community structure. Nutrient availability, biochemical oxygen demand, temperature, salinity and dissolved oxygen all play significant roles in explaining the variation observed in both algal community structure and abundance (Tookwinas & Songsangjinda 1999). Transparency measurement is routinely used as an approach to measure phytoplankton in commercial ponds. In the present study, Secchi disc depth and microalgae cell count were useful approaches for explaining the observed variation in chlorophyll levels in the reservoir tanks. Some authors suggest that Secchi depth should only be used as a simple visual index of the clarity of a body of water (Carlson & Simpson 1996). Others have found strong relationships between Secchi depth, chl-*a*, and total phosphorus concentrations,

suggesting the use of Secchi depth as a surrogate measure of algal chlorophyll or algal blooms (Carlson 1977). Phytoplankton community changes in shrimp ponds should be an additional tool to help manage shrimp ponds. Paying careful attention and establishing research focused on the influence of physical and chemical variables that are strongly related to phytoplankton community structures could provide aquaculturists the knowledge to better understand and best manage their ponds to optimize beneficial algal species and avoid problematic species.

Shrimp farms operate in all hemispheres and thus are situated in different environments and operate under different water quality, culture systems and fertilization programs. Thus, determination of the relationship between the microalgae community and chlorophyll levels in combination with Secchi depth and nutrient inputs could be a useful practice to establish a baseline of data. From this database, trends could be assessed to potentially enable early detection of any significant variation in the status of the ponds when relating the contribution of microalgae to the nutritional balance of shrimp in comparison with supplemented feed.

In summary a predominance of 76.3% Cyanobacteria (54% *Oscillatoria*) was observed for most of the nine months in all 12 semi-closed intensive shrimp ponds. However, with an adequate fertilization program in the reservoir tank, 60.7% Diatoms (52.7% *Chaetoceros* sp.) and 22.8% Cyanobacteria (22.6% *Oscillatoria* sp.) predominated. In the reservoir tank, total chlorophyll followed the pattern of cell count and Secchi depth independent of the type of predominant microalgae.

Conclusion

In conclusion, the microalgae composition was manipulated with a fertilization regimen in the reservoir tank to be different than that in the commercial shrimp ponds. Secchi depth and microalgae cell count are closely related to chlorophyll levels in a control environment and could be used as a reliable measurement to study the dynamics of the water column. Experimental results from a controlled environment should be compared to commercial scale systems, in order to determine the specific management of the fertilization program that will enhance diatom proliferation.

CHAPTER III

DIETARY EFFECT OF SQUID AND FISH MEAL ON GROWTH AND SURVIVAL OF PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI* IN THE PRESENCE OR ABSENCE OF PHYTOPLANKTON IN AN INDOOR TANK SYSTEM

Introduction

Global shrimp culture commonly experiences variations in price of feed ingredients (Coutteau, Ceulemans, Meeuws, Van Halteren, Robles & Nur 2008), largely due to demand and availability. The physical and nutritional quality of prepared feeds and efficacy of feed management are important because supplemental feeds can represent 20 - 50% of variable production costs, depending on the intensity of the culture system (Akiyama, Dominy & Lawrence 1992; Lawrence & Houston 1993; Tacon & Barg 1998). Thus, development of more cost-effective feeds with improved management is required for further development of the shrimp farming industry.

Natural productivity plays an important role in shrimp nutrition and needs to be considered when formulating shrimp feeds (Leber & Pruder 1988; Moss & Pruder 1995; Otoshi *et al.* 2001; Michele *et al.* 2004). Juvenile shrimp reared in organically rich, hyper-eutrophic water and fed two commercial diets, grew 50 - 73% faster than shrimp fed an identical diet but maintained in well water devoid of natural productivity (Leber & Pruder 1988). Growth enhancement has been attributed to the assimilation by shrimp of microalgae and microbial-detrital aggregates present in pond water (Moss & Pruder 1995). Moss, Pruder, Leber & Wyban (1992) showed that water from traditional shrimp

production ponds containing high levels of organic matter (i.e., microalgae) increased the growth of cultured shrimp by as much as 53%. The greater white shrimp growth in the presence of biofloc including microalgae versus clear water has also been previously evaluated (Tacon *et al.* 2002; Burford, Thompson, McIntosh, Bauman & Pearson 2004; Cuzon, Lawrence, Gaxiola, Rosas & Guillaume 2004; Moss, Forster & Tacon 2006; Wasieleski, Atwood, Stokes & Browdy 2006). However, no one has previously reported that the level of marine animal meals can be reduced in shrimp diets in the presence of microalgae.

The most common marine animal meals in shrimp feeds are fish meal, squid meal and krill meal (Lim & Dominy 1990; Akiyama & Dominy 1991; Tacon 1993; Tacon & Barg 1998). However, all three of these ingredients are natural supply-limited aquatic resources and thus relatively high priced. Efforts should be made to determine the best relative levels of these critical sources of protein in shrimp diets using feed performance and cost, especially considering availability of intrinsic sources of nutrition such as phytoplankton. Forster, Dominy, Lawrence, Castille & Patnaik (2010) evaluated growth and survival of *Litopenaeus vannamei* in a 35-day growth trial with 25 different combinations of squid, krill and fish meal in an indoor recirculating system in the relative absence of natural productivity. Growth of shrimp was greater when fed diets containing 11.6% fish and 22.9% squid meals as compared to a combination of 5.8, 9.7, 11.6, 14.5, 17.4, 13.5% of fish meal with 7.6, 11.4, 15.2, 19.1, and 22.9% of squid meal. These combinations, in the presence of and absence of krill meal resulted in similar growth, indicating that krill meal was not required with those levels of fish and squid in

a clear-water system. The growth response to different dietary levels of fish and squid meal in the presence of phytoplankton was not evaluated. In order to optimize diets for shrimp in green-water systems, it is critical to evaluate the potential of microalgae to reduce fish and squid meal inclusion levels in commercial feeds for *L. vannamei*. The objectives of the present study were: 1) to determine the effect of fish and squid feed levels in the presence and absence of microalgae on growth performance and survival of juvenile *L. vannamei*; and (2) to evaluate whether the presence of microalgae in the shrimp culture environment has the potential to reduce inclusion levels of squid and fish meals in commercial feeds.

Materials and Methods

This study was conducted in a greenhouse at the Alicorp Aquarium System (AAS), located at a shrimp farm in Tumbes, on the north Pacific coast of Peru. Specific-pathogen-resistant (SPR) *L. vannamei* postlarvae in their fourteenth day of development after metamorphosis (PL_{14}), were obtained from the Lobo Marino N°1 Laboratory (Salinas, Ecuador) and maintained in a greenhouse nursery system for 24 days prior to use. The nursery system was a high density polyethylene (HDPE)-lined, sediment-free wooden tank system (28 m^3 volume). Aeration was provided by regenerative blower. The PL_{14} (0.02 g, 2% CV) were stocked at a density of 65 per m^2 and manually fed 20 g of a commercial 40% crude protein (CP) crumble of 0.3 - 0.8-mm diameter (Nicovita® PC-1 40% crude protein; Alicorp, Lima, Peru) three times daily at 6:00, 12:00, and 18:00 h. Newly-hatched live *Artemia* sp. nauplii were also fed daily (50 nauplii per PL day^{-1}) for 2 weeks. For the next 10 days, only a commercial 40% CP crumble of 0.8 - 1.5

mm diameter (Nicovita® KR-1 40% crude protein; Alicorp, Lima, Peru) was fed at a rate of 45 g three times (6:00, 12:00 and 18:00 h) daily until harvest. This conditioning period allowed for acclimation to laboratory conditions (temperature 30.8^{o}C ± 1.07 SD and salinity 18.1 g L^{-1} ± 0.4 SD) and achievement of sufficient body weight for initiation of the experimental trial. Juveniles used in the trial were netted and transferred to the experimental units and allowed to acclimate for 1 week to a commercial 35% CP diet of 2 x 2 mm (length x diameter) (Nicovita® Alicorp, Lima, Peru - Bag Tag: min 35% protein, min 5% fat, max 12% moisture, max 4% fiber and max 12% ash) prior to starting the trial. The shrimp were netted and individually weighed to determine the initial weight before the 8-week trial.

The experimental system consisted of 36 indoor rectangular fiberglass aquaria (40 L volume; 0.1-m^2 bottom surface area) connected to either of two water supply systems: 1) a 2,680-L semi-closed recirculating system, consisting of a sump tank, three mechanical 200-, 75- and 5-µ filters, a biosphere biological filter and UV sterilizer and 2) an open system consisting of four, 28-m^3 wooden PVC-lined reservoir tanks. The water supply, which was also used for the shrimp farm ponds, was pumped from the "El Venado" estuary, through a 280-µ filter bag into four, 28-m^3 wooden PVC-lined reservoir tanks. The stock water was chlorinated to minimize the introduction of pathogens from wild vectors, and kill plankton and benthos. A dose previously proven effective of 10 mg L^{-1} of calcium hypochlorite solution (65% active ingredient) was used to provide a free chlorine residual concentration of 4 mg L^{-1} 30 min post-application with a targeted residual chlorine level of 1 mg L^{-1} after 24 h (analysis HACH-DR 2800,

Colorado, USA). After 72 h of aeration, the chlorine concentration of the water was reduced to < 0.05 mg L^{-1} and was used to fill 18 aquaria of the semi-closed recirculating system, referred to hereafter as the CLEAR water system. Two of the four chlorinated reservoir tanks were maintained in bloom with microalgae through a continuous fertilization program. The water was not seeded with microalgae. It bloomed from natural air spores in the reservoir water, as typically occurs in commercial lined ponds. Natural light source was used to maintain the algae. The water was fertilized with N:P and N:Si ratios of 15:1 and 1:1.25, respectively. Inorganic fertilization was applied at mid-day (12:00 ± 2 h) with NUTRILAKE® (15% nitrogen), Triple Super Phosphate (20% phosphorus) and NUTRICIL® (23% silicate). Thirty (30) g of ground 35% crude protein commercial feed (5.6% nitrogen, 0.85% phosphorus) and 30 g of commercial organic fertilizer (2.1% nitrogen, 0.65% phosphorus) were also added daily, as sources of carbon, nitrogen and phosphorus. After day 7, no NUTRILAKE® and Triple Super Phosphate were added. The source of metasilicate, NUTRICIL®, was maintained at a rate of 140 g day^{-1} (the equivalent of 50 kg ha^{-1} day^{-1}) throughout the trial. Ground commercial diet (Nicovita® 35% CP, Alicorp, Lima, Peru) and a commercial organic fertilizer (Nicovita® FB 12% CP, Alicorp, Lima, Peru) were maintained at the same rate of 30 g day^{-1} throughout the trial. This water was used to fill another 18 aquaria and was labeled the GREEN water system. The CLEAR water system had a 21% new water exchange rate (1.1-L $shrimp^{-1}$ day^{-1} - incoming chlorinated water that passed through 20- and 5-μ filters) and a recirculating rate of 0.95 L min^{-1} $tank^{-1}$, (3409% exchange $tank^{-1}$

day^{-1}). The GREEN water system was exchanged 20% (1.0-L shrimp^{-1} day^{-1}) daily for 6 days per week and 50% (2.5-L shrimp^{-1} day^{-1}) on the seventh day of the week.

Eight (equivalent to 80 shrimp m^{-2}) *L. vannamei*, very similar in size, were stocked in each aquarium. The mean weight per aquarium, weighed as a group, varied from 1.56 g to 2.56 g, with no significant differences among treatments (P = 0.964). Aeration was provided to each aquarium by one 2.5 x 2.5 x 5 cm air stone connected to a 0.5-hp air blower to maintain oxygen at a minimum of 6 mg L^{-1}. A light:dark photoperiod of 12:12 h was provided by supplemental compact fluorescent lighting. The distance between the white fluorescent lights (36 W) and the water in the aquarium system was 1.60 m.

Six experimental isonitrogenous (35% crude protein) and isocaloric (17.5 kJ g^{-1}) diets were formulated to contain one of three levels of squid meal (SM - 5%, 10% or 20%) combined with one of two levels of fish meal (FM - 6.5% or 12%) (Table 3.1 – The diets will be referred to hereafter as 5S6.5F; 5S12F; 10S6.5F; 10S12F; 20S6.5F; 20S12F with S and F representing squid and fish meals, respectively). De-hulled and de-fatted soybean meal was added to maintain equal protein and energy levels in experimental diets. At the Nicovita® pilot feed mill plant (NPP, Lima, Peru), all ingredients were finely ground at 250 ± 100 μ, mixed, steam conditioned, pelletized (compacted and cut), dried and cooled in order to obtain a fast sinking and water stable 2 x 2 mm pellet. Proximate analyses and pepsin digestibility (AOAC 971.09, 2005) were determined on each experimental diet. Diets where placed in labeled plastic containers and placed into a refrigerator at 4°C prior to feeding.

The biochemical composition of the diets was determined as follows: crude protein (DUMAS method; ISO 16634-1:2008**;** protein combustion analyzer LECO TruSpec®TRSCNC; Mugford 2000); crude fat (AOAC 920.39 C, 2005 Ether Extract); crude fiber (AOCS Ba 6-84, 1998); ash (AOAC 942.05, 2005); moisture (NTP-ISO 6469, 2002); nitrogen-free extract (100 – (Crude Protein + Crude Fiber + Ether Extract + Ash)); gross energy (Parr 6200 Oxygen Bomb Calorimeter, Parr, Moline IL, USA); amino acid concentrations (AOAC, 2000, No. 988.15); and phospholipids (AOCS Ca 12 - 55 2003 - Phosphorus). Prior to HPLC (high pressure liquid chromatography) analysis of amino acid concentration, duplicate feed samples (100 mg) were hydrolyzed in vacuum with 5 ml of HCl 6N for 22 h at 112 °C.

The feeding trial was conducted by feeding each experimental diet in triplicate over a period of 56 days using a randomized block design. Automated feeders were used to feed shrimp five times daily (08:00, 11:00, 14:00, 17:00 and 20:00 h) with equal rations at each feeding. The feed from the automatic feeder fell only in a specially designed 10-cm diameter plastic feed tray, which allowed daily removal and weighing of uneaten feed. Feces and molts were also removed daily. Feeding rates were based on a feed table beginning at 0.13 g feed shrimp^{-1} day^{-1} and gradually increased to a maximum of 0.42 g shrimp^{-1} day^{-1}. Feeding rate was always above satiation for all treatments. Feed rations were adjusted on a weekly basis, according to a projected weight gain of 2 g shrimp^{-1} week^{-1} and survival.

Table 3.1 Feed ingredients included in diets used in growth experiments. Values represent percent of total diet (Calculated on an as-fed basis)

Feed Ingredient	Diet designation					
	5S6.5F	5S12F	10S6.5F	10S12F	20S6.5F	20S12F
Squid Meal[1]	5	5	10	10	20	20
Fish Meal[1]	6.5	12	6.5	12	6.5	12
Soybean Meal[1]	39.77	34.31	34.78	29.54	20.04	12.48
Wheat[1]	33.7	33.7	33.7	33.5	38.5	40.6
Lecithin[1]	4	4	4	4	4	4
Fish oil[1]	2	2	2	2	2	2
Crustacean Meal[2]	2	2	2	2	2	2
Brewers Yeast[9]	2.7	2.7	2.7	2.7	2.7	2.7
Marine Salt[8]	1.5	1.5	1.5	1.5	1.5	1.5
Calcium Carbonate[8]	0.5	0.5	0.5	0.5	0.5	0.5
Potassium phosphate db[8]	0.5	0.5	0.5	0.5	0.5	0.5
Potassium Chloride[8]	0.75	0.75	0.75	0.75	0.75	0.75
Magnesium Oxide[8]	0.35	0.35	0.35	0.35	0.35	0.35
DL-Methionine[3]	0.18	0.14	0.17	0.11	0.11	0.07
Cholesterol[4]	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin Premix[5]	0.2	0.2	0.2	0.2	0.2	0.2
Mineral Premix[6]	0.1	0.1	0.1	0.1	0.1	0.1
Stay C 35%[7]	0.05	0.05	0.05	0.05	0.05	0.05

[1]Alicorp, Lima, PE: Peruvian giant squid muscle meal (*Dosidiscus gigas*), Peruvian fish meal (*Engraulis ringens*) and fish oil (*Engraulis ringens*), Dehulled and defatted soybean meal, liquid lecithin and hard red winter wheat.
[2] *Cervimunida johni* - BAHIA SpA, Santiago, Chile
[3]Sigma Chemical Company, Cleveland, Ohio, USA
[4]Dishman, Utrecht, The Netherlands

Table 3.1 Continued
[5]DSM Vitamin premix: Vit. A 10000 IU g^{-1}; B1 30 mg kg^{-1}; B2 15 mg kg^{-1}; DL Ca panthotenate 50 mg kg^{-1}; B6 35 mg kg^{-1}; B12 40 mcg kg^{-1}; Ascorbic acid 150; mg kg^{-1}, K3 3 mg kg^{-1}; D 33500 IU g^{-1}; E 150 IU g^{-1}; niacin 100 mg kg^{-1}; folic acid 4 mg kg^{-1}; biotin 1000 mcg kg^{-1}
[6]DSM Mineral premix: Mn, 40 mg kg^{-1}; Zn, 40 mg kg^{-1}; Cu, 25 mg kg^{-1}; Fe, 100 mg kg^{-1}; Se, 0.3 mg kg^{-1}; I, 0.35 mg kg^{-1}
[7]DSM, Lima, Peru
[8]ICN Biomedicals, Inc. Aurora, OH, USA
[9]Inactive *Saccharomyces cerevisiae* - ICC, Sao Paulo, Brazil

With the exception of salinity (g L^{-1}) that was measured during the afternoon, water temperature (°C), dissolved oxygen (mg L^{-1}), and pH were monitored twice daily (6:00 and 18:00 ± 0.5 hours) in the experimental aquaria and sump tank. Total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and alkalinity were monitored weekly in 24 experimental aquaria and the sump tank. The 24 aquaria were randomly chosen every week. Species identification of microalgae and cell density (cell mL^{-1}) were determined twice weekly and chlorophyll (mg m^{-3}) analysis was performed weekly in the GREEN water aquaria. Samples were collected at 12:00 hours using 500-mL plastic bottles. Subsamples (~150-mL) were preserved by adding 2 mL of Lugol's solution (Throndsen 1978). Cells were allowed to settle for 10 hours and subsequently processed for taxonomic and cell count analysis using an improved Neubauer hemocytometer at 600x magnification. Methodology for cell counts followed that of Venrick (1978). Malca's (1997) manual was used to separate microalgae into taxonomic divisions. Total chlorophyll was measured according to Strickland and Parsons (1972).

Crude protein (nitrogen x 6.25), essential amino acids, crude fat, ash, gross energy and moisture of phytoplankton samples were also determined. These samples

were collected once weekly, filtering approximately 180 L of the system incoming water containing primary productivity, with a set of 100-, 75- and 5-μ mesh filters for 2 minutes, then storing samples in a freezer (-12 to -18°C). The frozen samples were freeze-dried at -50°C for 48 h for reduction of moisture to less than 10%. Eight freeze-dried samples were combined and analyzed as two composite samples in order to verify accuracy of results.

Nutritional responses of the shrimp to the experimental diets were evaluated using the following indicators: (1) total weight gain (final mean wet weight – initial mean wet weight); (2) survival (final number of animals / initial number of animals) x 100; and (3) FCR - feed conversion ratio (total feed intake in dry weight basis / total gained biomass). The microalgae percent contribution to shrimp growth for each treatment also was estimated by comparing the shrimp weight gain in the GREEN versus CLEAR water systems ((GREEN mean wet weight gain - CLEAR mean wet weight gain) / CLEAR mean wet weight gain)*100.

A 2 x 3 x 2 factorial analysis of variance (ANOVA) was used to determine significant differences and their interaction among treatments (two fish meal levels, three squid meal levels and two microalgae levels) on weight gain and survival of shrimp. Survival was transformed by arcsine square root before being submitted to statistical analyses, to comply with the assumption of data being normally distributed. The normality was tested with “Kolmogorov-Smirnov”. When significant ($\alpha = 0.05$) F values were obtained, differences among treatments were determined with Fisher’s least

significant difference (LSD) multiple range test. The data were analyzed using the SPSS statistical software version 16 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

Proximate composition of the experimental diets (Table 3.2) varied slightly from the formulated values for crude protein and gross energy although they were constant in all diets. The desired levels of lipid, ash and fiber were achieved. Amino acid composition of the diets (Table 3.3) was also relatively constant.

There were no significant differences in treatments with respect to water quality indicators within each water system, with the exception of nitrite and alkalinity ($P<0.001$ - Table 3.4).

The level of phytoplankton in the CLEAR water system was negligible throughout the feeding trial and contrasted with that of the GREEN water system. Mean cell counts of microalgae among aquaria were not significantly different (ANOVA: $P>0.05$) in the GREEN water system (Fig. 3.1). Cyanobacteria were the predominant microalgae division, followed by Pyrrophytes, Diatoms and Chlorophytes (Table 3.5). Phytoplankton cell counts among dietary treatments were not significantly different (ANOVA: $P>0.05$) and had similar distribution patterns based on the average values of each aquaria in the GREEN water system (Fig. 3.2). The proximate and amino acid composition of composite microalgae samples were rather similar (Table 3.6).

Factorial ANOVA indicated that weight gain of shrimp in the GREEN water system was significantly higher and that FCR was significantly lower (Table 3.7). However, survival was not significantly different among dietary treatments and water systems. In all cases the interaction between the three factors (Fish meal, Squid meal and productivity) was not statistically significant (Table 3.7). No statistical differences in weight gain and FCR were evidenced in shrimp fed diets containing the various combinations of fish meal and squid meal, cultured in the CLEAR (with no phytoplankton) and GREEN water system (Table 7). Survival of shrimp among all water systems was not significantly different. Shrimp FCR values for those in the GREEN water system was significantly lower (ANOVA: $P<0.001$) among dietary treatments. The percent contribution of phytoplankton to weight gain of shrimp were 35% (5S6.5F), 47% (5S12F), 52% (10S6.5F), 28% (10S12F), 57% (20S6.5F) and 36% (20S12F).

Table 3.2 Nutrient composition of the experimental diets (values expressed on dry-matter basis in % unless otherwise stated)[a]

Component	Diet designation					
	5S6.5F	5S12F	10S6.5F	10S12F	20S6.5F	20S12F
Crude Protein	39.7	40.0	40.8	39.6	42.3	43.0
Crude Fat	9.4	9.4	9.7	9.2	9.8	9.4
Ash	10.1	7.8	10.5	10.1	10.0	8.0
Crude Fiber	2.0	2.3	1.9	2.0	2.1	2.2
[b]NFE	38.8	40.5	37.1	39.1	35.8	37.4
Gross Energy (kJ g^{-1})	18.2	18.5	18.2	18.3	18.1	18.7
Phospholipids	3.7	3.4	3.4	3.8	3.9	3.8
[c]Digestibility	94.3	93.1	93.2	94.0	93.7	93.8

[a]Values represent averages of duplicate samples
[b]Nitrogen-free extract
[c]Pepsin

Table 3.3 Amino acid composition (% of total) and essential amino acid index of experimental diets[a] (dry-matter basis)

Amino acids	Diet designation					
	5S12F	5S6.5F	10S12F	10S6.5F	20S12F	20S6.5F
Non essential amino acids						
Aspartate + Asparagine	4.1	4.3	4.2	4.1	4.1	4.2
Serine	1.8	1.9	1.9	1.8	1.8	1.9
Glutamate+Glutamine	6.9	7.3	7.1	6.9	6.9	7.1
Glycine	2.1	2.0	2.2	2.1	2.5	2.5
Alanine	1.9	1.9	2.1	1.9	2.2	2.2

Table 3.3 Continued

Amino acids	Diet designation					
	5S12F	5S6.5F	10S12F	10S6.5F	20S12F	20S6.5F
Proline	2.2	2.3	2.3	2.2	2.3	2.4
Tyrosine	1.2	1.2	1.1	1.3	1.2	1.2
Total NEAA	20.1	20.9	20.8	20.3	21.0	21.6
Essential amino acids						
Histidine	1.2	1.3	1.3	1.2	1.3	1.3
Arginine	3.0	3.0	3.1	3.0	3.3	3.4
Threonine	1.6	1.7	1.7	1.6	1.8	1.9
Valine	1.9	2.0	2.0	1.9	2.1	2.1
Methionine	0.8	0.9	0.9	0.9	1.1	1.1
Methionine+cysteine	1.4	1.5	1.7	1.6	1.9	2.0
Lysine	2.5	2.5	2.6	2.5	2.7	2.7
Isoleucine	1.8	1.9	1.9	1.8	2.1	2.1
Leucine	2.8	3.0	3.0	2.9	3.2	3.3
Phenylalanine	1.8	1.9	1.9	1.8	1.9	2.0
Total EAA	18.0	18.8	19.2	18.2	20.1	20.6
Total AA	38.1	39.7	40.0	38.6	41.1	42.2
EAA/Total AA (%)	52.8	52.7	52.2	52.7	51.1	51.1

[a] Values represent averages of duplicate samples

Table 3.4 Water-quality values from the GREEN water system (GWS) and the CLEAR water system (CWS) during the feeding trial

Parameter	GWS	CWS	*P*
Temperature (°C)	30.4 ± 1.07[a]	30.4 ± 0.98[a]	0.11
Salinity (g L^{-1})	25.5 ± 0.94[a]	25.5 ± 0.98[a]	0.827
pH	7.8 ± 0.13[a]	7.8 ± 0.14[a]	0.43
Dissolved Oxygen (mg L^{-1})	6.0 ± 0.22[a]	6.0 ± 0.21[a]	0.228
Chlorophyll (*ug* L^{-1})	12.1 ± 6.8		
TAN (mg L^{-1})	0.017 ± 0.01[a]	0.016 ± 0.01[a]	0.69
NO_2-N (mg L^{-1})	9.4 ± 5.96[a]	0.5 ± 0.23[b]	<0.001
Alkalinity (mg L^{-1})	100.4 ± 13.45[a]	93.0 ± 11.95[b]	<0.001

Values are means ± standard deviation of daily and weekly determinations over the 8-week trial.
n = 2016 for temperature, pH and DO; 1008 for salinity; 96 for ammonia, nitrite and alkalinity; 126 for chlorophyll.
Means within rows with the same letter are not significantly different (LSD α =0.05).

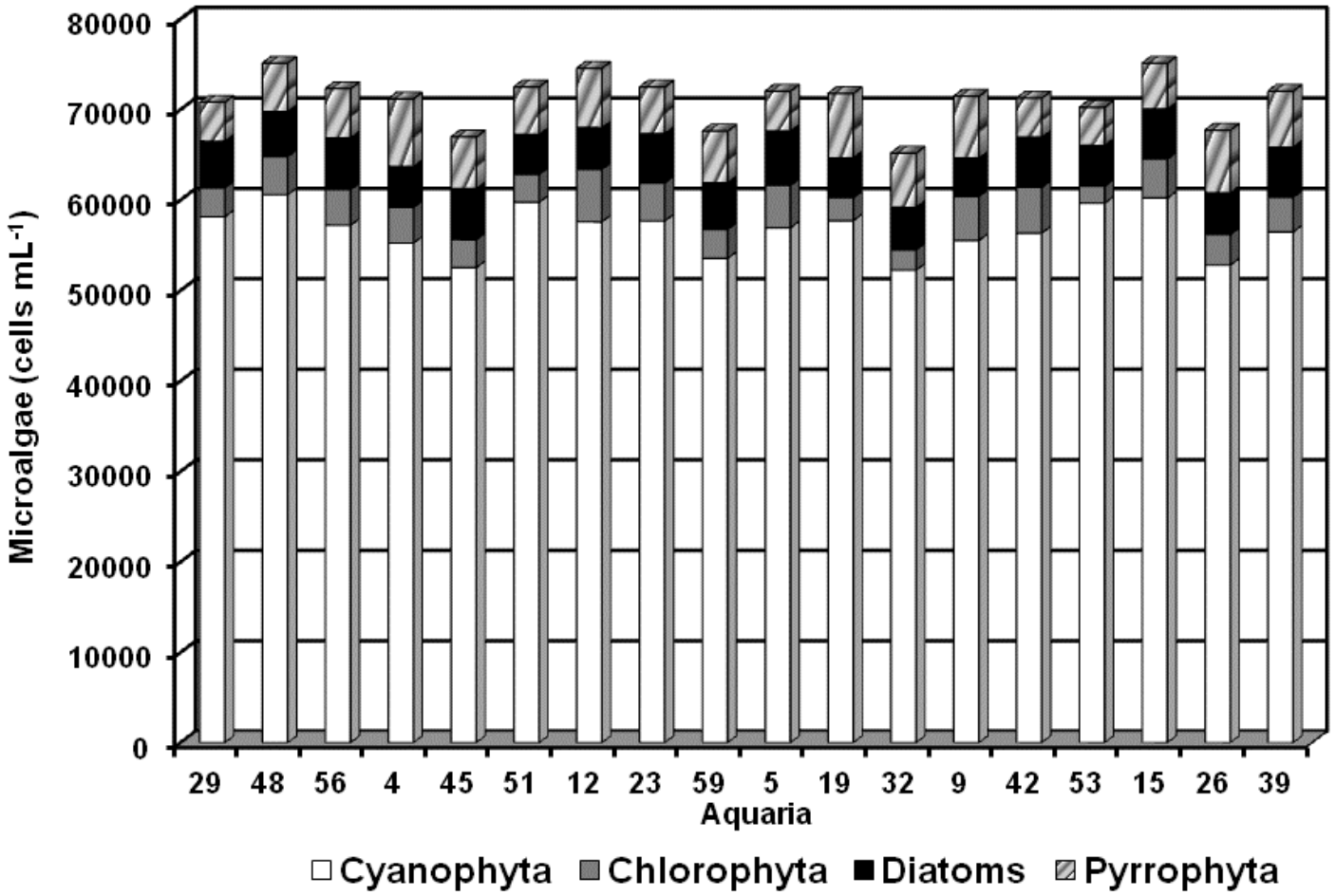

Figure 3.1 Microalgae mean cell count by divisions among aquaria (n=16: 2 samples, 8 weeks) in the GREEN water system. There were no significant differences among aquaria (ANOVA: $P > 0.05$).

Table 3.5 Percent count (cell ml^{-1}) of each microalga genera identified in the GREEN water system

Division	Genus	Frequency (%)
Diatoms	*Chaetoceros*	27.3
	Navicula	20.9
	Nitzchia	18.8
	Cymbella	21.9
	Thalassiosira	6.0
	Gyrosigma	3.6
	Amphiprora	1.0
	Amphipleura	0.5
	% Total Division	7.0
Cyanobacteria	*Oscillatoria*	100.0
	% Total Division	79.6
Pyrrophytes	*Phacus*	70.9
	Glenodinium	29.1
	% Total Division	8.1
Chlorophyts	*Chlamydomonas*	96.2
	Oocysts	3.8
	% Total Division	5.4

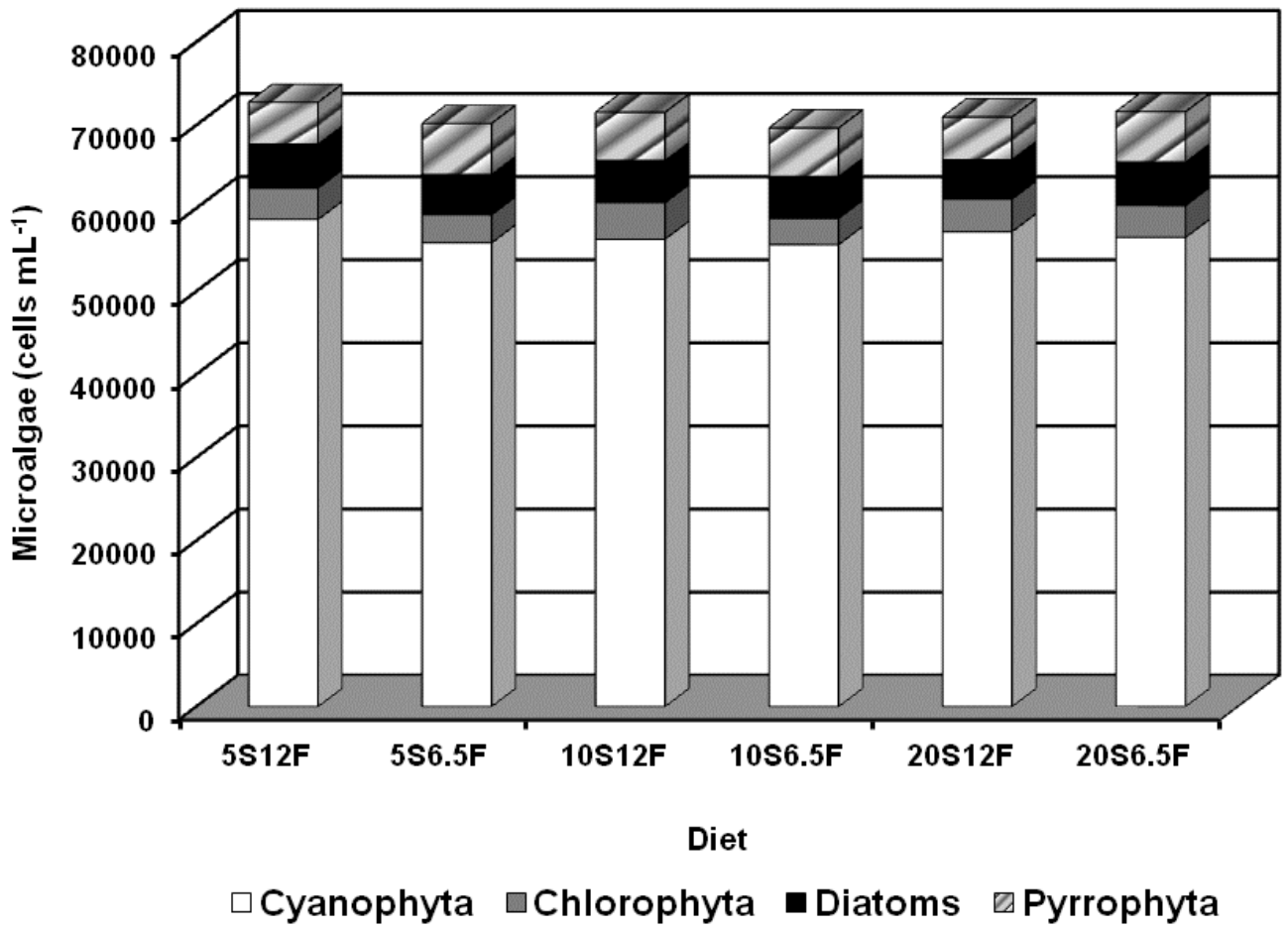

Figure 3.2 Microalgae mean cell count by divisions among dietary treatments (n=48: 2 samples, 8 weeks, 3 replicates) in the GREEN water system. There were no significant differences among treatments (ANOVA: $P > 0.05$).

Table 3.6 Protein, dry matter, lipids, ash, energy and essential amino acid of the microalgae used in the experiment (eight samples pooled into two composites – dry-matter basis)

Analyte	Composite	
	1	2
Dry matter (%)	5.8	6.2
Crude protein (%)	15.3	14.9
Crude Fat (%)	0.3	0.4
Ash (%)	45.6	45.9
Gross Energy (kJ g^{-1})	7.5	7.7
Essential amino acids (% of dry matter)		
Histidine	0.18	0.18
Arginine	0.72	0.71
Threonine	0.67	0.68
Valine	0.78	0.77
Methionine	0.26	0.25
Methionine + cysteine	0.39	0.37
Lysine	0.62	0.61
Isoleucine	0.61	0.60
Leucine	0.93	0.92
Phenylalanine	0.64	0.63

Table 3.7 Growth, survival and feed conversion ratio (FCR) of *L. vannamei* fed diets with graded combinations of fish (FM) and squid meals (SM) in CLEAR and GREEN water systems. All values represent population means (n = 3 for all measurements)

SM level (%)	FM	Water system	Initial weight (g)	Weight gain (g)	Survival %	FCR
5	6.5	GREEN	2.2	12.3[a]	96	1.74[a]
5	12	GREEN	2.0	12.5[a]	83	1.98[a]
10	6.5	GREEN	1.8	12.2[a]	96	1.82[a]
10	12	GREEN	1.9	11.9[a]	92	1.96[a]
20	6.5	GREEN	2.0	12.9[a]	96	1.68[a]
20	12	GREEN	1.9	12.7[a]	100	1.66[a]
5	6.5	CLEAR	1.8	8.2[b]	100	2.32[b]
5	12	CLEAR	1.8	8.5[b]	96	2.54[b]
10	6.5	CLEAR	1.8	8.0[b]	83	2.77[b]
10	12	CLEAR	1.9	9.3[b]	92	2.45[b]
20	6.5	CLEAR	1.8	8.3[b]	92	2.59[b]
20	12	CLEAR	1.8	9.3[b]	84	2.92[b]
PSE[c]			0.063	0.75	0.21	0.43
				ANOVA: *P*-values		
Fish meal				0.254	0.265	0.369
Squid meal				0.473	0.908	0.695
Water System				<0.001	0.463	<0.001
Fish meal x squid meal x water system				0.632	0.224	0.307

Water system: level of microalgae in aquaria.
[c] Pooled standard error of treatment means = square root (MSE/*n*).
Means within columns with the same letter are not significantly different (LSD $\alpha = 0.05$).

Discussion

Mean NO_2-N for aquaria in the CLEAR water system were lower compared to those in the GREEN water system, probably due to the nitrifying bacteria present in the bio-filter of the clear recirculation water system. Alkalinity was slightly lower in the CLEAR water system, probably due to a reduction of calcium carbonate ($CaCO_3$) levels, caused by a relatively low new water exchange rate in the recirculation system. Despite the differences in nitrite and alkalinity levels among the water systems, all water quality factors were within acceptable ranges for survival and growth of *L. vannamei* (Wickins 1976; Van Wyk, Davis-Hodgkins, Laramore, Main, Mountain & Scarpa 1999; Lin & Chen 2003; Sowers, Young, Shawn, Isely, Jeffery, Browdy & Tomasso 2004; Handy, Samocha, Patnaik, Gandy & McKee 2004; Cohen, Samocha, Fox, Gandy & Lawrence 2005; Mishra, Samocha, Patnaik, Speed, Gandy & Abdul-Mehdi 2008) throughout the duration of the 8-week experiment.

In the present study, no diet-related differences in weight gain and FCR were observed in the CLEAR and in the GREEN water system. Forster *et al.* (2010) observed that the optimum dietary combination of marine animal meals in diets for *L. vannamei* in indoor, clear water tanks was 11.6% fish meal and 22.9% squid meal. Even though results from this study and the present one are not comparable due to differences in shrimp strain, initial weight, growth rate, water source, diet ingredients and conditions of the trial, trends concerning the levels of fish and squid meal inclusion in diets for *L. vannamei* were observed. Taking into consideration the assemblage of phytoplankton characterized under the experimental conditions of the present study, the most cost-

effective combination of ingredients was the diet containing 6.5% fish meal and 5% squid meal, suggesting that fish and squid meal levels in commercial feeds for *L. vannamei* can possibly be reduced in the presence of microalgae in the culture water.

The estimation of percent contribution of phytoplankton to weight gain of shrimp in this study ranged from 28 to 57% for *L. vannamei* stocked at 80 m^{-2}. Lawrence and Houston (1993) estimated 77 to 83% percent contribution of natural productivity versus prepared diets to weight gain of shrimp in pens and in earthen ponds stocked at 15 m^{-2} and 20 m^{-2}, respectively. Anderson, Parker & Lawrence (1987) and Parker, Anderson & Lawrence (1989) using a stable carbon isotope tracer technique obtained similar estimates of percent contribution of natural productivity for assimilation of organic carbon by *L. vannamei* at similar stocking densities (20 m^{-2}) with values ranging from 53 to 77% and 44 to 86% compared to prepared feed. Even though there were not significant differences in the reported percentage contribution between the two studies, the variation in ranges could have been due to the different tracers used and variations in the characteristics of the pond biota as they were conducted in different years. Moss *et al.* (1992) evaluated weight gain of *L. vannamei* in 40 m^2 tanks provided with selected solid fractions of pond water with biofloc. Their study estimated contributions of 53% for particles between 0.5 and 5 μ and an additional 36% for particles > 5 μ, resulting in a total contribution of 89%. Wasieleski *et al*. (2006) detected significant differences in growth and survival of juvenile *L. vannamei* fed two levels of protein (35% and 25%) and reared for 20 days (stocking weight of 1.82 ± 0.71 g) in 50-L plastic bins at a density of 300 m^{-2} within a raceway with and without natural productivity (zero- exchange

system with suspended microbial floc). Conceptually, as the stocking density and harvest biomass increase in an earthen pond, the percent contribution of natural productivity to the nutritional requirements of shrimp would decrease if all other factors did not change (Lawrence & Houston 1993). Further, using higher stocking densities in an aquarium with no soil, as in the present study, the contribution of the four divisions of microalgae for *L. vannamei* growth, would probably be relatively less. Thus, the relative contribution of natural productivity obtained in the present study indicates that the method can be used for estimating the contribution of microalgae to the weight gain of shrimp in aquaria systems.

As none of the analyzed nutrients were limiting in any of the diets, differences in growth within diets cannot be attributed to any of the analyzed microalgae nutrients. The greater weight gain in the presence of primary productivity was possibly due to either 1) the phytoplankton contribution to a specific but unknown nutrient(s) in the shrimp diet, 2) unknown growth factors or 3) by affecting some water quality or other system factor. Further studies are recommended in order to better understand the specific nutrient(s) that may contribute to the better growth of shrimp in the presence of microalgae.

The significant weight gain exhibited by shrimp fed the different dietary treatments in the GREEN water system could also have been related to the greater digestion of unknown microalgae nutrients through the stimulation of the digestive enzymatic system of the shrimp when exposed to a microalgal environment. Moss, Divakaran & Kim (2001) compared the digestive enzyme activity of *L. vannamei* (mean weight = 0.07 g, SD = 0.004 g) reared at 40 m^{-2} in well and pond water plastic tanks for

35 days and fed a commercial 45%-protein feed. These authors observed that specific activities of serine protease, collagenase, amylase, cellulase, lipase and acid phosphatase in the shrimp digestive gland were more than two times higher in pond water than in well water and that shrimp reared in pond water had significantly greater growth (4.9 g) compared to well water (0.97 g). Divakaran & Moss (2004) investigated whether there was any difference in the specific activity of laminarinase between shrimp grown in an indoor clear water system and an outdoor zero-water exchange shrimp culture system. Specific activity of laminarinase was nearly seven times higher in shrimp grown in the outdoor zero-water exchange system, compared to shrimp grown in the indoor clear water system, thereby indicating the possibility of substrate specificity and enhanced enzyme production. If, as this study suggests, laminarinase is present in the digestive gland of shrimp reared in the presence of microalgae, this enzyme may serve an important role in the digestion of microalgae nutrient(s) and other sources of beta-1,3-linked glucans present in shrimp pond water.

The data obtained in the present study suggest that the squid and fish meal levels in commercial feeds for *L. vannamei* can be reduced. Further, microalgae present in the culture system significantly improved weight gain and FCR of shrimp, thus potentially reducing the feed cost associated with shrimp production.

CHAPTER IV

DIETARY EFFECT OF FISH OIL AND SOYBEAN LECITHIN ON GROWTH AND SURVIVAL OF JUVENILE *LITOPENAEUS VANNAMEI* IN THE PRESENCE OR ABSENCE OF PHYTOPLANKTON IN AN INDOOR SYSTEM

Introduction

In the last 10 years the world fish oil annual production remained relatively steady varying annually from 0.85 to 1.4 million metric tons (Bimbo 2011). In contrast, aquaculture production has increased by 340% in the last 20 years, placing a fish oil demand as an ingredient in fish and shrimp feeds of 85 percent (FAO 2010). Thus, inclusion levels of fish oil in shrimp diets must be reduced to allow for sustainable industry growth (Naylor *et al.* 2009).

Some fatty acids and other lipid classes like phospholipids, sterols and carotenoids are essential dietary components for crustaceans (NRC 2011). In the case of marine organisms, polyunsaturated fatty acids (PUFAs) and especially highly unsaturated fatty acids (HUFAs) are essential to provide pre-formed in the diet (Kanazawa, Teshima & Tokiwa 1977; Kanazawa, Tokiwa, Kayama & Hirata 1977; Kanazawa, Teshima, Endo & Kayama 1978; Kanazawa, Teshima & Tokiwa 1979; Kanazawa, Teshima & Ono 1979; Kanazawa, Teshima, Tokiwa, Kayama & Hirata 1979; Kanazawa, Teshima & Endo 1979). Past nutritional studies with various crustaceans indicate that the best survival and weight gain responses are achieved when the dietary lipid level is between 5 and 8% (D'Abramo 1997). Deshimaru, Kuroki & Yone (1979)

found that 6% of a mixture of Pollock (*Pollachius vivens*) liver oil and soybean oil provided in a ratio between 3:1 and 1:1 was associated with comparatively higher growth and feed efficiency values for *P. japonicus*. Lim, Ako, Brown & Hahn (1997) reported that menhaden oil, rich in n-3 HUFAs, was the most nutritious source of lipid for *L. vannamei*, and among plant oils, those rich in linolenic (18:3n-3) had a higher nutritional value than those rich in linoleic (18:2n-6). Gonzales-Felix, Lawrence, Gatlin & Perez-Velazquez (2002) reconfirmed that menhaden oil showed a higher nutritional value for juvenile *L. vannamei* due to the higher levels of essential fatty acids (EFAs), particularly n-3 HUFAs.

One role of dietary phospholipids (PLs) in crustaceans is associated with the absorption (Lester, Carey, Cooperstein & Dowd 1975) and transport of lipids, especially cholesterol and triglycerides, in the hemolymph (Teshima, Kanazawa, Sasada & Kawasaki 1982; Kanazawa, Teshima & Sakamoto 1985; Teshima, Kanazawa & Kakuta 1986a,b,c; Kanazawa 1993, Teshima 1997). Crustaceans are able to synthesize PLs such as phosphatidylcholine (PC) from phosphorylcholine, diglycerides, and phosphatidylethanolamine (PE - Shieh 1969; Ewing & Finamore 1970). Teshima *et al.* (1986b) also suggested the conversion of dietary TG to PL classes such as PC and PE in the hepatopancreas of *M. japonicus*. It is also known that HUFAs are preferentially incorporated and conserved in the polar lipid fraction (which include the PL and glycolipid fractions) of crustacean tissue, and that specifically docosahexaenoic acid (DHA or 22:6n-3) and eicosapentaenoic acid (EPA or 20:5n-3) are precursors of PC and PE, respectively (Clarke 1970; Kanazawa *et al*. 1977; D'Abramo, Bordner, Dagget,

Conklin & Baum 1980; D'Abramo & Sheen 1993). Greater growth and lower feed conversion ratio values were reported by Gonzales-Felix *et al.* (2002) in *L. vannamei* juveniles fed diets with 3.1% of a commercial soybean lecithin (97.6% PLs) as compared to shrimp fed diets without lecithin. Akiyama *et al*. (1992) recommended a total PLs content of 2% of the diet for *L. vannamei* juveniles. Teshima *et al.* (1986a) reported that the percentage weight gain and feed efficiency (g gain/g feed) of *P. japonicus* juveniles were markedly lower when a PLs-deficient diet was fed compared to one supplemented with 3% soybean lecithin (67 % purity).

Gong, Lawrence, Dong-Huo, Castille & Gatlin (2000) reported an interaction between the requirements of dietary cholesterol (CH) and the presence of de-oiled soybean lecithin in the diet. In the absence of the lecithin ingredient, the dietary requirement of *L. vannamei* for CH was reported to be 0.35%. When dietary levels of the lecithin (97.6% purity) increased to 1.5% and 3.0%, the CH requirement correspondingly decreased to 0.14% and 0.13%, respectively. However, Chen (1993) and Chen & Jenn (1991) did not observe any interaction between the presence of PC (80% purity – levels of 0, 1.25, 2.5 and 5%) and the requirement for CH (levels of 0, 0.5 and 1%) as determined by weight gain response of *P. monodon*. The larger number of replicates and uniformity of shrimp growth with small variation within each dietary treatment could have been the reasons for the identification of interactions between dietary PLs and CH in the study of Gong *et al*. (2000).

Kontara, Coutteau & Sorgeloos (1997) reported a significant interaction between dietary PC and *n*-3 HUFAs in postlarvae of *P. japonicas* for resistance to osmotic stress,

proposing that PLs may possibly improve the utilization efficacy of EFAs supplied in the diet as neutral lipid, and thus reduce the quantitative requirements for *n*-3 HUFAs in shrimp diets. However, Gonzales-Felix *et al.* (2002) did not observe any interaction between PLs and EFAs based on weight gain of *L. vannamei* juveniles.

The nutritional value of microalgae depends primarily on its biochemical composition and specific nutritional requirements of the animal consuming it (Brown *et al.* 1989). Most micro-algal species contain reasonable concentrations of proteins, carbohydrates, EFAs, carotenoid pigments, minerals and vitamins for various aquatic animals (Takeuchi, Lu, Yoshizaki & Satoh 2002). PLs sub-fractions detected in most microalgae include phosphatidyl inositol (PI), phosphatidyl choline (PC), phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE) and diphosphatidyl glycerol (DG) (Ben-Amotz, Tornabene & Thomas 1985). Saturated fatty acids constitute about 15 to 30% of the total fatty acids in green microalgae; whereas, their range in diatoms and prymnesiophytes is 30 to 40%. Green microalgae also are relatively low in the mono-unsaturated fatty acids (5 to 20%) but high in the PUFAs (50 to 80%); whereas, prymnesiophytes and diatoms have similar levels of both the mono-unsaturated (20 to 40%) and polyunsaturated (20 to 50%) fractions (Brown *et al*. 1989).

The preceding suggests that 1) PLs may reduce dietary EFAs requirements and 2) microalgae are potentially a source of EFAs and/or PLs. For these reasons, it is important to acquire a better understanding of the contribution of various nutrients from sustainable feedstuffs to shrimp in the absence and presence of natural productivity, which will give information to optimize formulated shrimp diets and reduce inclusion

levels of fish oil. Thus, the objective of the present study was to evaluate weight gain, survival and feed conversion ratio (FCR) of *Litopenaeus vannamei* fed different dietary combinations of fish oil (FO) and 65% purity soybean lecithin (LT) in an indoor clear-water system and green-water system (presence of microalgae) to 1) determine if dietary PLs increases the availability of EFAs, and 2) evaluate if phytoplankton contributes to meeting the dietary requirements for PLs and EFAs, so that FO inclusion levels in commercial shrimp diets can be reduced.

Materials and Methods

The feeding trial was conducted in a greenhouse at Alicorp facilities, located at a shrimp farm (latitude 3°27'33.22"S, longitude 80°19'54.13"W – 3msl) in Tumbes, on the north Pacific coast of Peru. Specific-pathogen-resistant (SPR) *L. vannamei* post-larvae in their 12^{th} day of development after metamorphosis (PL_{12}), were obtained from the "Lobo Marino 2" Laboratory (Mar Bravo, Salinas, Ecuador – latitude 2°14'09.27"S, longitude 80°57'33.67"W – 3msl) and maintained in a greenhouse nursery system for 45 days prior to use in the experiment. The nursery system was one high density polyethylene (HDPE)-lined, sediment-free wooden tank (28 m^3 volume). Aeration was provided by regenerative blower. The PL_{12} (0.02 g, 1.8% CV) were stocked at a density of 172 and manually fed 12 g of a commercial 0.6- to 0.8-mm crumble feed (Nicovita® PC-1 40% crude protein; Alicorp, Lima, Peru) six times a day (8:00, 10:00, 12:00, 14:00, 18:00 and 20:00 h) for the first week. Also, during this first week, newly-hatched live *Artemia* sp. nauplii (50 nauplii per larvae day^{-1}) were fed twice a day (06:00 and 14:00 h). For the second week, 12 g of the crumble feed were fed six times a day (6:00, 9:00, 12:00,

15:00, 18:00 and 21:00 h). Then, for the next 19 days, 33 g of a commercial 0.8- to 1-mm crumble feed (Nicovita® KR-1 40% crude protein; Alicorp, Lima, Peru) were fed five times a day (6:00, 10:00, 14:00, 18:00 and 22:00 hours) until the harvest. This conditioning period allowed for acclimation to laboratory conditions (temperature 30.6°C ± 0.6 SD and salinity 34.3 g L^{-1} ± 0.7 SD), and achievement of a desired body weight for initiation of the experimental trial. Juveniles used in the trial were netted and transferred to the experimental units and allowed to acclimate for 1 week to a commercial 35% CP diet of 2 x 2 mm (length x diameter) (Nicovita® Alicorp, Lima, Peru - Bag Tag: min 35% protein, min 5% fat, max 12% moisture, max 4% fiber and max 12% ash) prior to starting the trial. The shrimp were then netted and individually weighed to determine the initial weight before the 8-week feeding trial.

The experiment was conducted in two greenhouse-enclosed water systems (CWS and GWS). The CWS consisted of 30 indoor rectangular fiberglass aquaria (40-L volume; 0.1-m^2 bottom surface area), connected to a 2,680-L semi-closed recirculating system and one, 28-m^3 wooden PVC-lined reservoir tank. The GWS which provided natural productivity (i.e., microalgae) consisted of 24 wooden PVC-lined tanks (1,944-L volume; 1.72 m^2 bottom surface area; 1.13 m depth), connected to an open system that consisted of three, 28-m^3 wooden PVC-lined reservoir tanks. Each aquarium was provided with one 1.5 cm x 1.5 cm x 3 cm air stone connected to a 0.5-HP air blower; whereas, each tank was provided with ten 2.5 x 80 cm airlifts connected to two 2.0-HP air blowers. A light: dark photoperiod of 12:12 h was provided in the CWS by supplemental compact fluorescent (36 W) lighting. The distance between the white

fluorescent lights and the water was 1.60 m. Natural photoperiod was provided in the GWS. The water supply for both culture systems, also used for the LATIMAR shrimp farm ponds, was initially pumped from the "El Venado" estuary, through a 280-micron filter bag into the six 28-m^3 wooden PVC-lined reservoir tanks enclosed in a separate greenhouse. The water was chlorinated to minimize the introduction of pathogens from wild vectors, reduce organic matter and kill plankton and benthos. A dose previously proven to be effective (10 mg L^{-1} of calcium hypochlorite solution - 65% active ingredient) was used to provide a free chlorine residual concentration of 4 ppm 30 min post application with a targeted residual chlorine level of 1 ppm after 24 h. After 72 h of water aeration, the chlorine concentration was below 0.05 ppm, such that this clear water was used for filling the 60 aquaria. The CWS had a daily exchange rate of 25% new water (1.4-L $shrimp^{-1}$ day^{-1}) and a recirculating rate of 0.95 L min^{-1} $aquarium^{-1}$ (3409% exchange $aquarium^{-1}$ day^{-1}).

For the GWS, three of the six reservoir tanks were allowed to develop phytoplankton blooms through a continuous fertilization program. This water with primary productivity was used to fill the 1,944-L tanks in which the first 6 weeks the exchange rate was 10% (1.4-L $shrimp^{-1}$ day^{-1}) every 2 days. During the following 2 weeks of the trial, 20% (2.8-L $shrimp^{-1}$ day^{-1}) of the water was exchanged also every 2 days.

Both CWS and GWS were stocked with the same density of *L. vannamei* (equivalent to 80 shrimp m^{-2}). Eight shrimp were stocked in each aquarium, while 138 shrimp where stocked in each tank. The individual mean weight in the CWS varied from

3.9 to 5.4 g, while the weight in the GWS varied from 5.2 g to 6.1 g, with no significant differences among treatments (CWS: P = 0.983; GWS: P = 0.689). Thus, both systems were stocked with a size large enough to achieve linear growth rate to the termination of the experiment.

Six experimental isonitrogenous (35% protein) diets were formulated with technical grade feedstuffs (Table 4.1), to contain one of three levels of crude Peruvian fish oil – FO (10, 20 or 30 g kg^{-1}) combined with one of two levels of soybean lecithin (65% purity) – LT (10 or 40 g kg^{-1}) (Table 4.1). Crude fat and energy levels were allowed to vary among the diets. All ingredients were obtained from commercial sources (Table 4.1 footnotes). At the Nicovita® pilot feed mill plant (NPP, Lima, Peru), all dry ingredients were finely ground and mixed for 10 min. Fluid ingredients were then added and the slurry was mixed for an additional 10 min. The mixture was then steam conditioned at 90 ± 2 °C and pelletized (compact and cut) with a pellet mill through a 2.5 x 5-mm die. Pellets were cooled and oven - dried at 73 °C under constant air for 45 min. in order to obtain a fast-sinking and water-stable pellet. Proximate analysis, essential amino acid composition, essential fatty acid composition, cholesterol, astaxanthin and pepsin digestibility (AOAC 971.09, 2005) were determined on each experimental diet. Diets where bagged in labeled plastic containers and placed into a refrigerator at 4°C prior to feeding.

The biochemical composition of the diets was determined as follows: crude protein using the DUMAS method (ISO 16634-1:2008; protein combustion analyzer LECO TruSpec®TRSCNC; Mugford 2000). Crude fat content of the diets was

determined gravimetrically following extraction of lipids according to the Soxhlet method (AOAC 920.39 C, 2005). Gross energy was determined by an adiabatic bomb calorimetry (Parr 6200 Oxygen Bomb Calorimeter, Parr, Moline IL, USA). Standard procedures were used for moisture, crude fiber (AOCS Ba 6-84, 1998), ash (AOAC 942.05, 2005), moisture (NTP-ISO 6469, 2002), PLs (AOAC Ca 12-55, 2003 - Phosphorus), pepsin digestibility (AOAC 971.09, 2005), EFAs composition by gas liquid chromatography (GLC) (Marine Oils Modified, AOCS Ce-1b-89, 2003), amino acid composition by high pressure liquid chromatography (HPLC) (Waters AccQ Tag), cholesterol (AOAC 994.10 modified) and astaxanthin (DSM version - 1.4 - 02.05.05). Prior to HPLC analysis of amino acid concentrations, duplicate feed samples (100 mg) were hydrolyzed in vacuum with 5 ml of 6N HCl for 22 h at 112 °C.

The proximate, essential amino acid, EFAs, PLs and astaxanthin contents of phytoplankton also were determined. Samples of phytoplankton were obtained by filtering approximately 180 L of water per tank once a week from four tanks with a set of 100-μ, 75-μ and 5-μ mesh screens and then storing in a freezer (-12 to -18°C). Frozen samples were transported to a laboratory located 800 km south from the trial facility. Upon arrival, samples were thawed, centrifuged and freeze-dried at -50°C for 48 h to allow moisture to be reduced to less than 10%. Samples were then pooled into two composite samples per system per week to have enough material for analyses.

Table 4.1 Composition of experimental diets containing different levels of soybean lecithin and fish oil. Values represent percent of total diet (Calculated on as-fed basis, g/100 g)

Feed Ingredient	Fish oil – Lecithin level (%)					
	1 – 1	1 - 4	2 - 1	2 - 4	3 – 1	3 – 4
Squid meal[1]	10	10	10	10	10	10
Fish meal[1]	12	12	12	12	12	12
Soybean meal[1]	25	25	25	25	25	25
Wheat[1]	44	41	43	40	42	39
Soybean lecithin[1]	1	4	1	4	1	4
Crude fish oil[1]	1	1	2	2	3	3
Yeast[8]	2.3	2.3	2.3	2.3	2.3	2.3
Marine salt[7]	2	2	2	2	2	2
Vitamin premix[2]	0.2	0.2	0.2	0.2	0.2	0.2
Mineral premix[3]	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol[4]	0.05	0.05	0.05	0.05	0.05	0.05
Mold inhibitor[6]	0.15	0.15	0.15	0.15	0.15	0.15
Calcium carbonate[7]	0.5	0.5	0.5	0.5	0.5	0.5
Potassium phosphate db[7]	0.5	0.5	0.5	0.5	0.5	0.5
Potassium chloride[7]	0.75	0.75	0.75	0.75	0.75	0.75
Magnesium oxide[7]	0.35	0.35	0.35	0.35	0.35	0.35
DL-Methionine[9]	0.12	0.12	0.12	0.12	0.12	0.12
Vitamin C[5]	0.05	0.05	0.05	0.05	0.05	0.05

[1]Alicorp, Lima, PE.: Peruvian squid muscle meal (*Dosidiscus gigas*), Peruvian fish meal (*Engraulis ringens*) and fish oil (*Engraulis ringens*), dehulled and defatted soybean meal, liquid lecithin (65% purity), hard red winter wheat flour and wheat middling.
[2]DSM Vitamin premix: Vit. A 10000 IU g^{-1}; B1 30 mg kg^{-1}; B2 15 mg kg^{-1}; DL Ca panthotenate 50 mg kg^{-1}; B6 35 mg kg^{-1}; B12 40 mcg kg^{-1}; Ascorbic acid 150; mg kg^{-1}, K3 3 mg kg^{-1}; D 33500 IU g^{-1}; E 150 IU g^{-1}; niacin 100 mg kg^{-1}; folic acid 4 mg kg^{-1}; biotin 1000 mcg kg^{-1}

Table 4.1 Continued
[3]DSM Mineral premix: Mn, 40 mg kg^{-1}; Zn, 40 mg kg^{-1}; Cu, 25 mg kg^{-1}; Fe, 100 mg kg^{-1}; Se, 0.3 mg kg^{-1}; I, 0.35 mg kg^{-1}
[4]Dishman, Utrecht, The Netherlands basis.
[5] DSM (L-ascorbyl-2-polyphospate, 35%).
[6]MoldZap Degussa, Lima, Peru.
[7]ICN Biomedicals, Inc. Aurora, OH, USA.
[8]Inactive *Saccharomyces cerevisiae* - ICC, Sao Paulo, Brazil.
[9]Sigma Chemical Company, Cleveland, Ohio, USA.

The feeding trial was conducted over a period of 56 days (8 weeks) by feeding shrimp in five replicate aquaria per dietary treatment in the CWS. The same six experimental diets were simultaneously evaluated in the GWS in four replicate tanks. Concurrent feeding trials allowed comparison of the contribution of phytoplankton to the shrimp's growth. Automatic feeders (wheel-type in CWS and belt type in GWS) were used to feed shrimp five times per day (6:00, 10:00, 14:00, 18:00 and 22:00 h) with equal rations at each feeding. The feed from the automatic feeder fell only in a specially designed 10-cm diameter plastic feed tray in the case of CWS and 60-cm diameter in GWS, which allowed daily removal and weighing of uneaten feed. Only in the CWS were feces and molts removed daily, while bottom tank accumulation of sludge in the GWS was completely flushed on a daily basis. Feeding rates were based on a feed table beginning at 0.13 g $shrimp^{-1}$ day^{-1} and gradually increased to a maximum of 0.42 g $shrimp^{-1}$ day^{-1}. Feeding rate was above satiation and was adjusted on a weekly basis, according to theoretical average weight and survival in the case of the CWS, and to the determined average sample weights and theoretical survival in the GWS. Uneaten feed was collected with feed trays adapted in size for each system and weighed daily.

With the exception of salinity (g L^{-1}) that was measured at noon, water temperature (°C), dissolved oxygen (mg L^{-1}), and pH, were monitored twice daily (6:00 and 18:00 ± 00:30 h) using a YSI 85® Meter and a YSI pHYSI10 Ecosense® pen-style meter (YSI Inc., Yellow Springs, OH), in the CWS and GWS. Total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and alkalinity were monitored weekly. Luminosity and Secchi depth (cm) were measured at midday in the GWS using a Secchi disk (± 0.01m) and a light meter Photometer SM 700 Milwaukee®, respectively. Incidence of luminosity was measured following the method of Walker *et al*. (2007) in three representative sections. Identification of microalgae, cell density (cell mL^{-1}) and chlorophyll (mg m^{-3}) analysis was performed twice a week only in the GWS. Samples were collected at 12:00 hours using 1000-mL beakers. Subsamples (~150-mL) were preserved by adding 2 mL of Lugol's solution (Throndsen 1978). Cells were allowed to settle for 10 hours and subsequently processed for taxonomic and cell count analysis using an improved Neubauer hemocytometer at 600x magnification. Methodology for cell counts followed that of Venrick (1978). Malca's (1997) manual was used to separate microalgae into taxonomic divisions. Total chlorophyll was measured according to Strickland and Parsons (1972).

After termination of the feeding trial, the cephalothorax (fore and midgut gland) and abdominal muscle (with no intestine) of 50 shrimp per tank or eight shrimp per aquarium were separated and wrapped in aluminum paper, packed in plastic bags, labeled and stored in a freezer (Electrolux 7.6 cu ft, FE22, PR) at -12 to -18°C for 1 week. Frozen samples were transported to a laboratory located 800 km south from the

trial facility. Prior to analysis, the cephalothorax and abdominal muscle were removed and stored at -86°C in an ultra low temperature freezer (DFU-014 Giant Star, Gyeonggi-do, Korea) for 24 h. Composite samples of 25 or four shrimp (cephalothorax or abdominal muscle) from the GWS or CWS, respectively, were vacuum freeze-dried at -50°C for 24 h in the dark with a 4.5-L freeze-drier (FreeZone Benchtop, Labconco Corp., MO, USA). The dried cephalothorax and abdominal muscle were finely ground with a grinder (Retsch ZM 200 Retsch GmbH, Haan, Germany), and stored at -86°C for analysis. Dried samples were analyzed in duplicate for total lipids (AOAC 920.39 C, 2005), essential fatty acid composition by GLC (Marine Oils Modified AOCS Ce-1b-89, 2003), phospholipids (AOAC Ca 12-55, 2003 - Phosphorus) and astaxanthin (DSM version - 1.4 - 02.05.05) to compare the shrimp tissue variations among dietary treatments in the absence and presence of primary productivity.

Nutritional responses of the shrimp to the experimental diets were evaluated using the following indicators: (1) total weight gain (final mean wet weight – initial mean wet weight); (2) survival [(final number of animals / initial number of animals) x 100]; and (3) FCR - feed conversion ratio (total dry weight feed intake / total gained biomass). The microalgae percent contribution to shrimp growth for each treatment also was estimated by comparing the shrimp weight gain in the GWS versus CWS ((GWS mean wet weight gain - CWS mean wet weight gain) / CWS mean wet weight gain) x 100.

A 2 x 3 factorial analysis of variance (ANOVA) was used to determine significant differences and their interaction among treatments (two LT levels and three

FO levels) on weight gain and survival of shrimp. When significant ($\alpha = 0.05$) F values were obtained, differences among treatments were determined with Fisher's least significant difference (LSD) multiple range test. The data were analyzed using the SPSS statistical software version 16 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

Values of pertinent water quality characteristics obtained in the CWS and GWS during this experiment were within acceptable levels, suggesting shrimp were maintained under desired water quality conditions for the duration of the 8-week trial (Table 4.2 and 4.3). Luminosity in the GWS varied for the three different locations depending on the day light and amount of shadow from the clouds.

Crude protein values (Table 4.4) of the experimental diets varied slightly from the formulated values. Amino acid and cholesterol composition of the diets were very similar. Crude fat, EFAs, PLs, neutral lipids and gross energy varied with the FO and LT inclusion levels in the experimental diets (Tables 4.4 and 4.5). Proximate composition (Table 4.6) of microalgae showed relatively high levels of ash (45.02%), protein (22.7%) and astaxanthin (4.5%) compared to the lipid levels.

Table 4.2 Water quality values during the feeding trial in the clear water system (CWS)

	TEMP (°C)	OXYGEN (mg L^{-1})	pH	SALINITY (g L^{-1})	NH_3-N (mg L^{-1})	NO_2-N (mg L^{-1})	ALKALINITY (mg L^{-1})
Number	7800	7800	7800	3900	333	333	333
MEAN	30.5	5.9	8.0	31.6	0.04	1.6	94.7
MAX	32.2	7.1	8.2	35.2	0.1	8.3	130
MIN	28.8	4.9	7.7	27.7	0.0	0.3	73.0
Std. DEV	0.7	0.3	0.1	2.5	0.04	1.6	10.9

Table 4.3 Water quality values during feeding trial in the green water system (GWS)

	TEMP. (°C)	OXYGEN (mg L^{-1})	pH	SALINITY (g L^{-1})	NH_3-N (mg L^{-1})	NO_2-N (mg L^{-1})	ALKALINITY (mg L^{-1})	Secchi Depth (cm)	Chl *a* (mg m^{-3})	Chl *b* (mg m^{-3})	Chl *c* (mg m^{-3})	Luminosity (light cm^{-2})
Number	3024	3024	3024	1512	216	216	216	1512	432	432	432	63
MEAN	31.3	5.6	8.0	31.7	0.0	6.2	115	50	19.2	0.5	3.5	34611
MAX	33.8	6.8	8.4	34.1	0.02	28.4	140	97	64.1	6.5	12.6	219400
MIN	28.6	4.7	7.6	27.7	0.0	0.0	73	24	0.9	0.0	0.0	4208
Std. DEV	0.9	0.3	0.2	1.8	0.0	5.5	10.2	17	12.9	0.9	2.3	32425

Table 4.4 Analyses of proximate composition, cholesterol, energy and amino acid concentrations of the diets used in experiments (average of duplicate analyses)

Analyte	Fish oil – Lecithin level (%)					
	1 – 1	1 – 4	2 – 1	2 - 4	3 – 1	3 - 4
Dry matter (%)	9.8	12.1	10.1	11.3	10.1	11.6
Dry-matter basis (% unless otherwise stated)						
Crude protein	39.1	38.9	39.3	39.3	39.5	39.7
Total lipid	5.4	8.4	6.7	9.3	7.7	11.3
Ash	10.5	8.5	10.5	8.6	10.5	8.7
Fiber	3.4	1.7	3.7	2.0	1.8	1.6
Cholesterol	0.31	0.32	0.31	0.34	0.31	0.31
Gross energy (kJ g^{-1})	17.2	18.1	16.8	18.3	17.7	18.8
Pepsin Digestibility	94.3	94.1	95.7	95.4	95.1	95.8
Essential amino acids (% of diet)						
Arginine	2.50	2.53	2.57	2.54	2.51	2.56
Cystine	0.40	0.47	0.44	0.52	0.59	0.59
Histidine	1.00	1.03	1.01	1.03	1.02	1.02
Isoleucine	1.61	1.65	1.63	1.59	1.58	1.61
Leucine	2.80	2.87	2.85	2.75	2.76	2.79
Lysine	2.33	2.37	2.38	2.42	2.43	2.45
Methionine	0.85	0.90	0.90	0.90	0.90	0.86
Phenylalanine	1.68	1.72	1.73	1.66	1.67	1.70
Threonine	1.49	1.52	1.54	1.52	1.51	1.53
Valine	1.77	1.80	1.79	1.72	1.74	1.75

Table 4.5 Lipid, lipid class composition and source (lecithin: LT; fish oil: FO; other ingredients: OI) of phospholipids (PLs) and neutral lipid (NL) of diets used in experiments

Analyte	FO – LT Level (%)					
	1 – 1	1 – 4	2 – 1	2 - 4	3 – 1	3 - 4
Total lipid (%)	5.4	8.4	6.7	9.3	7.7	11.3
Total PLs (%)	1.7	3.5	1.8	3.5	1.7	3.9
PLs –> LT	0.7	2.6	0.7	2.6	0.7	2.6
PLs –> OI	1.1	0.9	1.2	0.9	1.1	1.3
NL –> LT	0.4	1.4	0.4	1.4	0.4	1.4
NL –> FO	1.0	1.0	2.0	2.0	3.0	3.0
NL –> LT + FO	1.4	2.4	2.4	3.4	3.4	4.4
NL –> OI	2.4	2.5	2.6	2.4	2.7	3.0
Essential fatty acids (% of diet)						
Linoleic (18:2n-6)	1.57	3.01	1.49	2.88	1.43	3.07
Linolenic (18:3n-3)	0.16	0.31	0.15	0.30	0.16	0.33
Arachidonic (20:4n-6)	0.04	0.04	0.05	0.05	0.07	0.07
EPA (20:5n-3)	0.48	0.53	0.74	0.76	0.97	1.08
DHA (22:6n-3)	0.31	0.34	0.42	0.43	0.50	0.57
LOA:LNA	10	10	10	10	9	9
EPA:DHA	1.5	1.5	1.8	1.8	2.3	1.9

Table 4.6 Proximate composition, lipid class, essential amino acid, fatty acid and astaxanthin concentrations of the microalgae pool obtained in the experiment (average of duplicate analyses)

Analyte	Microalgae
Dry-matter basis (% unless otherwise stated)	
Ash	45.0
Crude protein	22.7
Total lipid	1.1
Phospholipids	0.2
Astaxanthin	4.5
Gross energy (kJ g^{-1})	10.26
Essential amino acids	
Arginine	1.06
Cystine	0.35
Histidine	0.72
Isoleucine	0.97
Leucine	1.56
Lysine	0.96
Methionine	0.48
Phenylalanine	1.10
Threonine	1.11
Valine	1.17
Essential fatty acids	
Linoleic (18:2n-6)	0.127
Linolenic (18:3n-3)	0.21
Arachidonic (20:4n-6)	0.06
EPA (20:5n-3)	0.127
DHA (22:6n-3)	0.042

The level of phytoplankton in the CWS was negligible throughout the feeding trial as compared to the GWS. In the GWS, Diatoms were the predominant algal type; followed by Cyanobacteria, Chlorophytes and Pyrrophytes (Table 4.7). The lowest microalgae cell count was observed during the first week of the trial (Fig. 4.1). Microalgae cell count reached the highest levels during the second week, which was also the period during which the diatoms were also the highest. During week 3, the levels of diatoms decreased and Cyanobacteria increased. Microalgae cell counts, specifically diatoms, steadily increased from week 5 to 7. Phytoplankton cell counts among dietary treatments were not significantly different (ANOVA: $P>0.05$) and had similar distribution patterns based on the average values of each tank in the GWS (Fig. 4.2).

Increasing weekly levels of total chlorophyll were observed during the trial. Week 7 achieved the greatest mean chlorophyll level and week 1 the lowest mean value (Fig. 4.3). From week 3 to 7, the chlorophyll levels increased steadily, but reduced slightly in week 8. Chlorophyll *a* was predominant during the 8 weeks of the trial, followed by chlorophyll *c*; and finally chlorophyll *b* was measured at the lowest level.

Table 4.7 Percent count (cells ml^{-1}) of each microalgae genera identified in the tank system. n = 384 (6 treatments, 4 replicates, 2 samples per week)

Division	Genus	Frequency (%)
Diatoms	*Thalassiosira*	69
	Navicula	15.9
	Nitzchia	12.1
	Chaetoceros	1.9
	Cymbella	0.7
	Amphiprora	0.2
	Achnanthes	0.1
	Amphipleura	0.1
	% Total Division	70
Cyanobacteria	*Oscillatoria*	100
	% Total Division	19.7
Chlorophyts	*Chlamydomonas*	100
	% Total Division	7.9
Pyrrophytes	*Glenodinium*	61
	Phacus	30
	Peridinium	9.2
	% Total Division	2.4

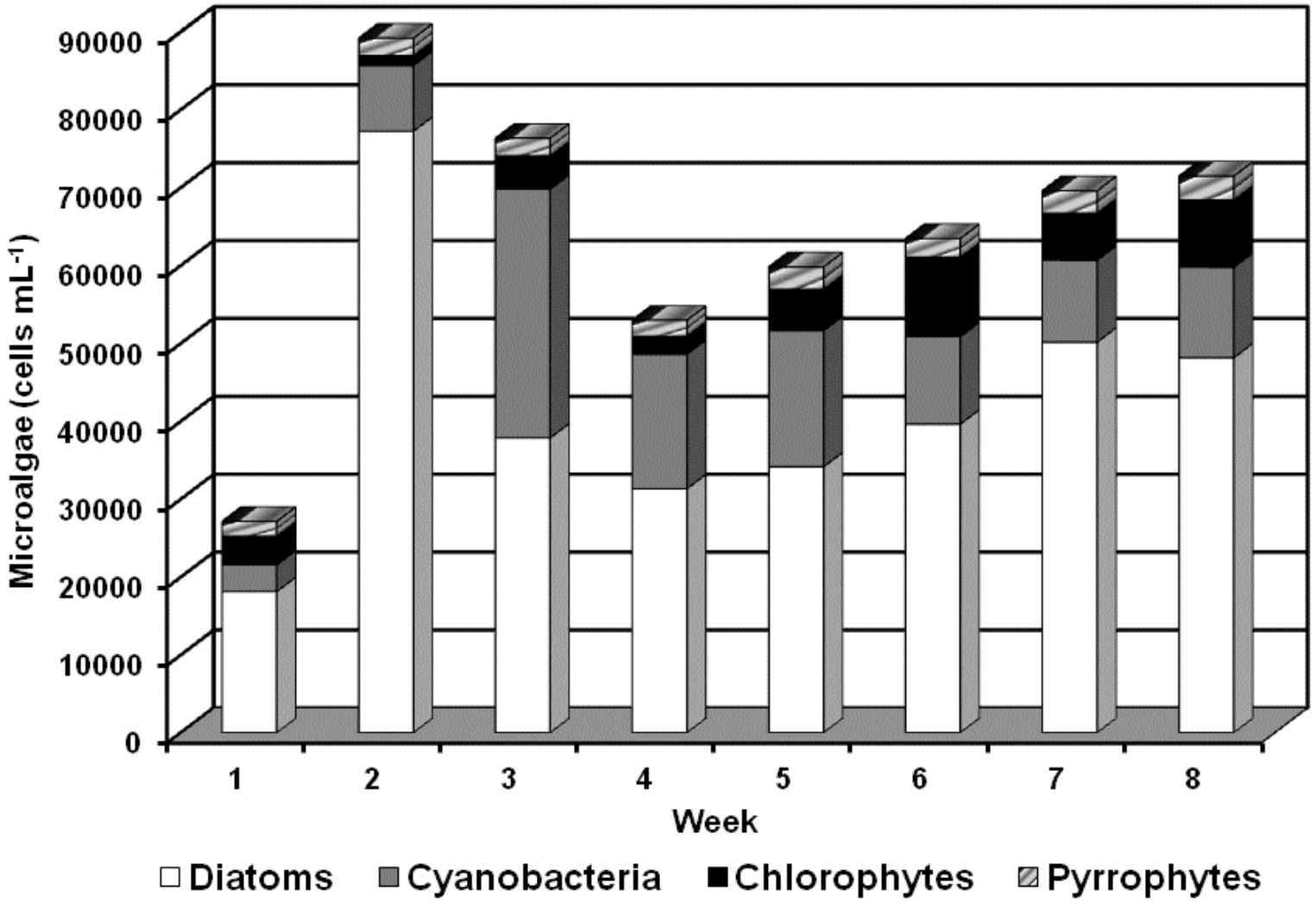

Figure 4.1 Weekly mean (n = 48) microalgae cell count divisions for the tank system.

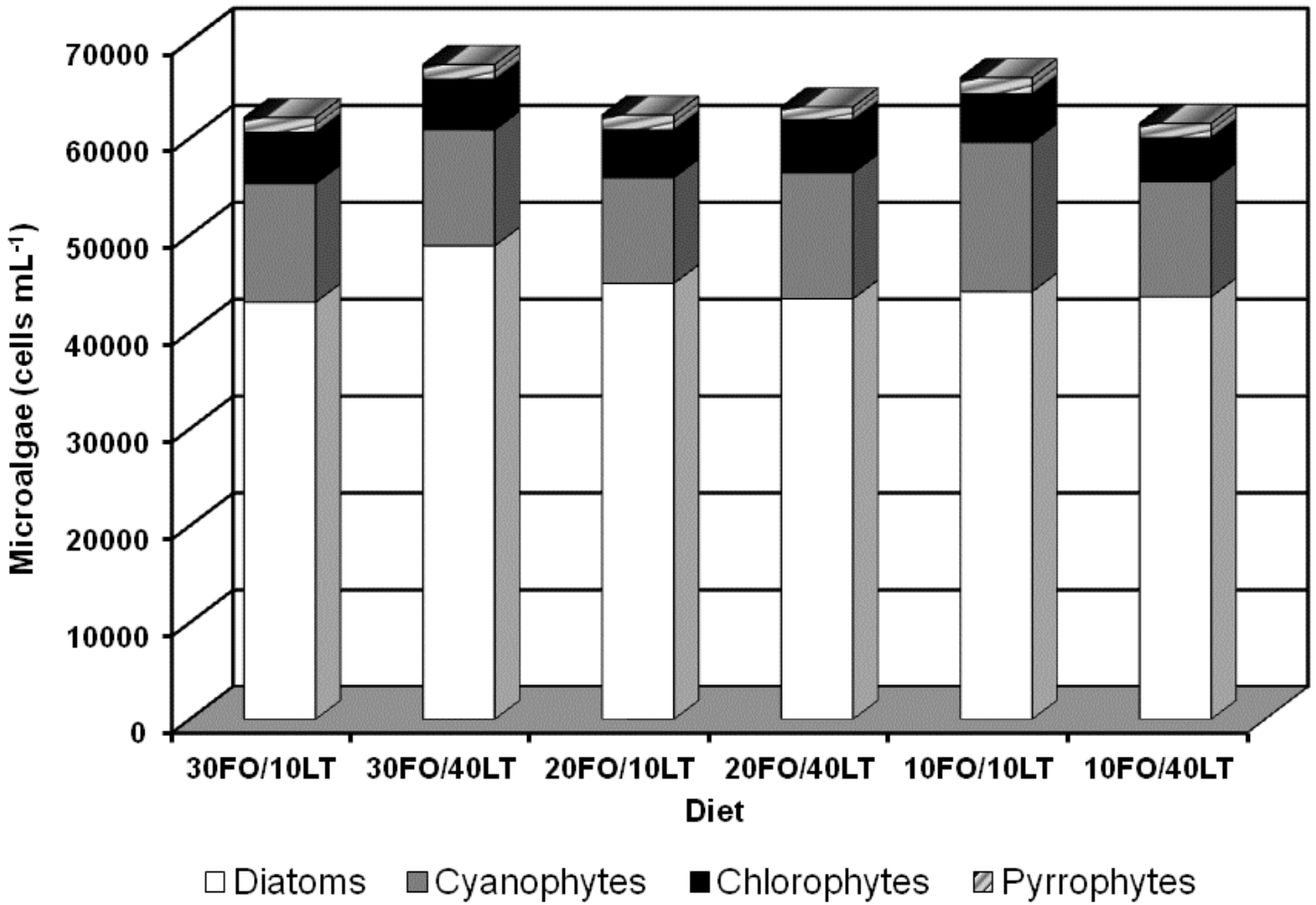

Figure 4.2 Microalgae mean cell count divisions among dietary treatments. 64 measurements per treatment (2 samples, 8 weeks, 4 replicates).

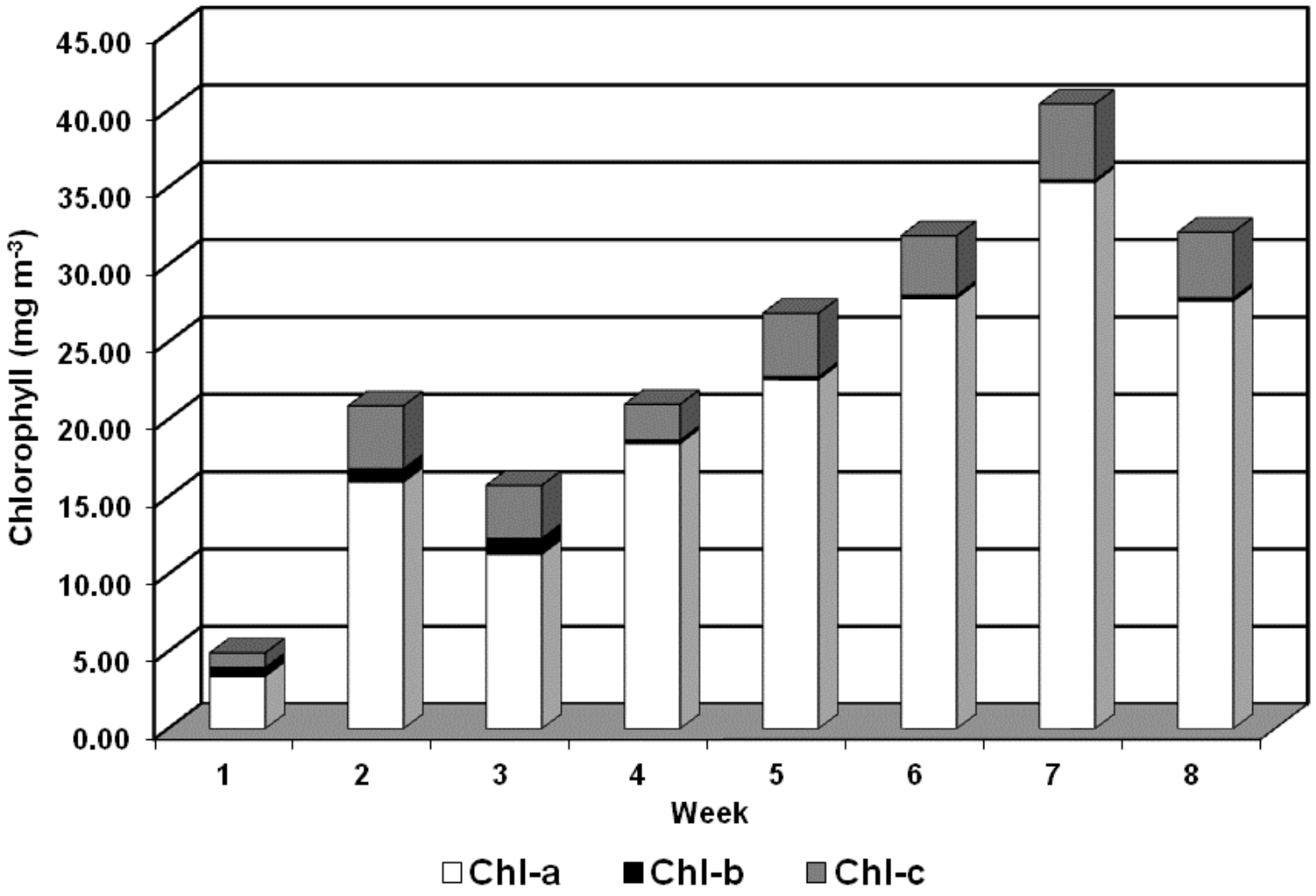

Figure 4.3 Weekly chlorophyll (mg m^{-3}) of microalgae (n = 48).

In both culture systems, the CWS and GWS, a significant main effect of LT level on weight gain and FCR was observed at the end of the trial. However, the interaction between the two factors (FO level and LT level) was not statistically significant (Tables 4.8 and 4.9). Significant differences ($P < 0.001$) in weight gain and FCR of shrimp evaluated in the CWS as compared to the GWS were observed. ANOVA indicated that weight gain of shrimp in the GWS was significantly higher and that FCR was significantly lower compared to the CWS. Shrimp fed diets containing 1% FO and 1% LT had significantly lower weight gain and higher FCR compared to the rest of the treatment diets with the exception of the diet containing 2% FO and 1% LT. However, survival was not significantly different among dietary treatments and water systems. The estimated percent contribution of phytoplankton to the weight gain of shrimp varied from 38.8 ± 6.0 % (2% FO and 4% LT) to 60.6 ± 13.5 % (1% FO and 1% LT).

For all the dietary treatments, shrimp cultured in the GWS showed significantly ($P<0.05$) higher mean lipid, linoleic acid (LOA), PLs and astaxanthin levels in the cephalothorax compared to those from the CWS (Table 4.10 and 4.11). However, in the muscle tissue the levels of lipid, PLs and linolenic acid (LNA), but not astaxanthin, were not different for all dietary treatments.

Total lipid in shrimp cephalothorax was seven (CWS) to 12 (GWS) times greater than that in muscle tissue (Table 4.11). However, PLs in shrimp cephalothorax was one (CWS) to three (GWS) times greater. Higher dietary LT significantly affected the lipid cephalothorax content in CWS and GWS ($P < 0.05$). In the case of PLs content in cephalothorax, the higher dietary LT level was reflected only in CWS ($P < 0.05$). Total lipid content in shrimp muscle tissue was not affected by dietary FO or LT (Table 4.10).

Table 4.8 Growth, survival and feed conversion ratio of *L. vannamei* fed diets with graded combinations of fish oil (FO) and lecithin (LT) in the GWS. All values represent population means (n = 4)

Diet	FO – LT level (%)	Initial weight (g)	Weight gain (g)	Survival (%)	FCR
Diet 1	1 - 1	5.6	14.3[c]	97.1	1.64[c]
Diet 2	1 - 4	5.6	15.8[a]	94.4	1.53[a]
Diet 3	2 - 1	5.5	14.8[bc]	95.1	1.62[bc]
Diet 4	2 - 4	5.5	15.4[ab]	93.7	1.59[ab]
Diet 5	3 - 1	5.6	15.2[ab]	95.1	1.57[ab]
Diet 6	3 - 4	5.6	15.8[a]	93.7	1.54[a]
PSE[c]		0.066	3.25	0.002	0.05
P-value		0.689	<0.001	0.363	0.098
		Means of main effect [h]			
FO level (%)					
1		5.6	15.0	95.7	1.58
2		5.5	15.1	94.4	1.60
3		5.6	15.5	94.4	1.56
LT level (%)					
1		5.6	14.7[c]	95.8	1.61[c]
4		5.6	15.7[d]	93.9	1.55[d]
		ANOVA: *P*-values			
FO Level		0.689	0.080	0.432	0.293
LT Level		0.754	< 0.001	0.068	0.023
FO x LT		0.978	0.057	0.827	0.339

[c] Pooled standard error of treatment means = square root (MSE/*n*).
[h] Main effect means followed by the same letter are not significantly different at $P = 0.05$ by Fisher's protected least-significant-difference procedure.
Means within columns with the same letter are not significantly different (Fisher's protected least-significant-difference, $\alpha = 0.05$).

Table 4.9 Growth, survival and feed conversion ratio of *L. vannamei* fed diets with graded combinations of fish oil (FO) and lecithin (LT) in the CWS. All values represent population means (n = 5)

Diet	FO – LT level (%)	Initial weight (g)	Weight gain (g)	Survival (%)	FCR
Diet 1	1 - 1	4.5	8.8[e]	100	2.07[e]
Diet 2	1 - 4	4.7	10.7[d]	100	1.73[d]
Diet 3	2 - 1	4.5	9.8[de]	100	1.88[de]
Diet 4	2 - 4	4.8	10.8[d]	100	1.72[d]
Diet 5	3 - 1	4.8	10.4[d]	100	1.77[d]
Diet 6	3 - 4	4.5	10.8[d]	100	1.70[d]
PSE[c]		0.14	0.92		0.03
P-value		0.983	0.02		0.012
		Means of main effect[h]			
FO level (%)					
1		4.6	9.7		1.9
2		4.6	10.3		1.8
3		4.7	10.6		1.7
LT level (%)					
1		4.6	9.7[f]		1.9[f]
4		4.7	10.7[g]		1.7[g]
		ANOVA: *P*-values			
FO Level		0.983	0.15		0.109
LT Level		0.569	0.005		0.004
FO x LT		0.257	0.229		0.190

[c] Pooled standard error of treatment means = square root (MSE/*n*).
[h] Main effect means followed by the same letter are not significantly different at $P = 0.05$ by Fisher's protected least-significant-difference procedure.
Means within columns with the same letter are not significantly different (Fisher's protected least-significant-difference, $\alpha = 0.05$).

Table 4.10 Analyses[a] of the lipid and phospholipids contents of tissues from shrimp fed various levels of fish oil (FO) and lecithin (LT) in the clear water system (CWS) and green water system (GWS) (n = 2)

FO - LT	Tissue	Lipid		Phospholipids	
Level (%)		CWS	GWS	CWS	GWS
1 – 1	CT[b]	6.35	12.89	2.15	4.18
	Muscle	2.81	3.08	1.76	2.32
1 – 4	CT	10.94	15.40	3.81	4.30
	Muscle	3.00	2.55	2.32	1.77
2 – 1	CT	9.53	12.10	2.89	4.25
	Muscle	2.93	2.78	2.13	1.57
2 – 4	CT	10.74	15.39	3.96	4.16
	Muscle	2.84	2.98	2.06	2.04
3 – 1	CT	8.59	14.69	2.37	4.72
	Muscle	2.86	3.08	2.00	1.89
3 – 4	CT	12.38	17.59	3.86	5.43
	Muscle	3.04	2.81	2.43	2.10

[a] Dry-matter basis (% unless otherwise stated).
[b] CT – Cephalothorax

Table 4.11 Analyses[a] of the essential fatty acids and astaxanthin content of tissues from shrimp fed various levels of fish oil (FO) and lecithin (LT) in the clear and green water system (n = 2)

FO - LT	Water	LOA		LNA		ARA		EPA		DHA		Astaxanthin (mg kg^{-1})	
Level (%)	System	CT[b]	Muscle	CT	Muscle	CT	Muscle	CT	Muscle	CT	Muscle	CT	Muscle
1 – 1	Clear	1.42	0.43	0.07	0.02	0.07	0.06	0.40	0.38	0.31	0.28	1.11	1.08
	Green	2.64	0.47	0.11	0.02	0.12	0.08	0.48	0.38	0.35	0.31	7.76	5.75
1 – 4	Clear	2.90	0.56	0.13	0.03	0.08	0.06	0.47	0.36	0.35	0.26	0.72	1.03
	Green	3.70	0.47	0.16	0.02	0.11	0.06	0.49	0.31	0.34	0.22	8.23	5.76
2 – 1	Clear	1.87	0.40	0.10	0.02	0.10	0.06	0.59	0.44	0.44	0.32	0.99	1.34
	Green	2.23	0.38	0.10	0.02	0.12	0.07	0.54	0.36	0.40	0.27	7.14	5.65
2 – 4	Clear	2.54	0.47	0.12	0.02	0.09	0.05	0.53	0.37	0.38	0.27	0.83	0.96
	Green	3.42	0.51	0.14	0.02	0.12	0.07	0.54	0.36	0.37	0.26	7.54	5.54
3 – 1	Clear	1.35	0.33	0.06	0.01	0.10	0.06	0.53	0.41	0.38`	0.30	1.09	1.09
	Green	2.16	0.38	0.08	0.02	0.13	0.08	0.57	0.41	0.38	0.30	7.06	5.95
3 – 4	Clear	2.55	0.44	0.11	0.02	0.10	0.06	0.58	0.41	0.42	0.30	0.73	1.05
	Green	3.89	0.42	0.18	0.02	0.15	0.07	0.72	0.34	0.50	0.25	7.39	5.34

[a]Dry-matter basis (percent unless otherwise stated).
[b]Cephalothorax.

The highest lipid level (CWS: 12.4%; GWS: 17.6%) was observed in cephalothorax of shrimp fed the diet containing 3% FO and 4% LT, while shrimp fed the diet with 1% FO and 1% LT had the lowest value (CWS: 6.4%; GWS: 12.9% - Table 4.10). Also, the fatty acid composition of the test diets was reflected to a certain extent in the fatty acid composition of the cephalothorax of the shrimp cultured in CWS and GWS (Table 4.11). For instance, significantly higher ($P < 0.05$) levels of LOA and LNA were observed in shrimp cultured in both systems fed diets containing 4% LT. However, no significant differences were observed in the fatty acid composition of shrimp muscle tissue, with the exception of DHA in shrimp cultured in the GWS.

Discussion

All of the water quality conditions throughout the duration of the 8 - week feeding trial in both the CWS and GWS were within recommended commercial production ranges for optimal growth and survival of *L. vannamei* (Samocha, Lawrence & Bray 1993). Measurements of NO_2-N, temperature and alkalinity were slightly lower in the CWS compared to those levels in the GWS. In the case of nitrite and alkalinity, these differences primarily could be attributed to the activity of nitrifying bacteria and calcium imbalance in the recirculating water with no phytoplankton. In the case of temperature, the greater volume of water and depth of the culture units in the GWS (1,944 L and 1.13 m depth) compared to the CWS (40 L and 0.45 m depth) could be the principal physical factors that made the water remain slightly warmer in the GWS.

Results of the present study showed that dietary FO levels could be reduced to 1% with the inclusion of 4% dietary LT (3.5% PLs), suggesting that dietary PLs may

improve the efficiency of EFAs utilization when supplied as neutral lipid. Beneficial effects could be related to an increased transport and lipid mobilization from the hepatopancreas to the hemolymph and to other tissues and organs, resulting in enhanced lipid deposition and increased energy availability for growth (Teshima *et al*.1986a,b; Teshima 1997). Studies on juvenile shrimp also have demonstrated that dietary PLs supplementation increased growth compared to diets without PLs (Teshima *et al*.; 1986a,b; Gong, Lawrence, Gatlin, Jiang & Zhang 2001; González-Félix *et al.* 2002; Kumaraguru, Ramesh & Balasubramanian 2005; Hu, Tan, Mai, Ai, Zhang & Zheng 2011). In addition, the fact that shrimp fed diets with higher levels of PLs and lower levels of HUFAs performed as well as those fed diets with higher HUFA levels was probably because liquid soybean lecithin may serve as a source of choline, inositol and EFAs or even energy (Coutteau, Geurden, Camara, Bergot & Sorgeloos 1997). In addition, dietary PLs have been shown to improve the properties of artificial diets by reducing the leaching of water-soluble nutrients, in particular manganese and B vitamins (Castell, Boston, Conklin & Baum 1991), and also may act as emulsifiers, facilitating the digestion and absorption of fatty acids, bile salts, and other lipid-soluble substances such as vitamins (Coutteau *et al*. 1997), thus contributing to the superior performance of shrimp.

The best weight gain responses to different dietary levels of one oil (Menhaden fish oil or Pollack liver oil) or a mixture of oils (Pollock liver oil + soybean oil; Soybean oil + Pollock residual oil + short-necked clam oil; Cod liver oil + corn oil) in shrimp (*P. japonicus*) and other crustaceans (*Homarus americanus*, *Procambarus acutus*,

Macrobrachium rosenbergii), are generally achieved at dietary levels between 5 and 8% (D'Abramo 1997; NRC 2011), and that levels higher than 10% often retard growth (Akiyama *et al*. 1992; Hu, Tan, Mai, Ai, Zheng & Cheng 2008), most probably due to a reduction in consumption caused by high caloric content and/or inability to metabolize high levels efficiently (NRC 2011). In the present study, the lipid levels of experimental diets varied from 5.2 to 10.8 % (dry-weight basis), which were within the recommended range. However, the diet containing 1% FO and 1% LT (1.5% PLs) showed the lowest lipid level, which could be one of the factors for the reduced weight gain compared to the rest of the experimental diets. In addition, the level of dietary triglyceride judged to be best is ultimately influenced by a variety of factors: the quality and quantity of dietary protein; the quantity, quality and availability of other energy sources and the oil source (D'Abramo 1997). For instance, in the present study, the better performance of shrimp fed 4% LT (3.5% PLs) and 1% FO compared to the diet containing 1% LT (1.5% PLs) and 1% FO, could have been due to a combined effect of 56% more lipids, 106% more dietary PLs, 92% more LOA and 94% more LNA and 5% more gross energy.

Survival in the present study, however, was not affected by the lower dietary levels of lecithin (1.5% PLs). According to Coutteau, Camara & Sorgeloos (1996), significant effects of PL supplementation on survival only has been demonstrated in larval *M. japonicus* (Kanazawa *et al*. 1985; Teshima *et al*. 1986c). Furthermore, all of the water quality conditions throughout the duration of the 8-week feeding trial were within recommended ranges for *L. vannamei* shrimp culture, which also may have contributed to high survival.

In the present study the fatty acid composition of the cephalothorax reflected, to a certain extent, that of the test diets. These results are in agreement with other studies in various shrimp species (*P. monodon*: Deering, Fielder & Hewitt 1997; Millamena 1989; Kumaraguru *et al.* 2005; *F. indicus*: Colvin 1976; *M. japonicus*: Guary, Kayama, Murakami & Ceccaldi 1976; Kayama, Hirata, Kanazawa, Tokiwa & Saito 1980; *L. vannamei:* González-Félix *et al.* 2002; Hu *et al*. 2011; Ju, Forster, Dominy & Lawrence 2011). In addition, studies have demonstrated retention of specific HUFAs when supplementing dietary saturated and monounsaturated fatty acids (Araujo & Lawrence 1991; Xu, Wenjuan, Castell & O'Dor 1994; Deering *et al.* 1997). However, in the present study, shrimp fed diets with higher levels of PUFAs showed no preferential incorporation of EPA or DHA.

The estimation of the contribution of phytoplankton from the data obtained in this study indicate that there was a contribution to weight gain of 38.8% ± 6.0 SD to 60.6% ± 13.5 SD for *L. vannamei* stocked at 80 m^{-2}. This greater weight gain observed in the GWS compared to the CWS is similar to the one reported in chapter III, indicating that both methods (Method A: CWS and GWS both shrimp reared in aquaria; Method B: CWS shrimp reared in aquaria and GWS shrimp reared in tanks) can be used to estimate the percent contribution of phytoplankton to the weight gain of shrimp.

The higher levels of PLs, LOA, LNA and astaxanthin contained in the cephalothorax of shrimp cultured in the GWS compared to the ones cultured in the CWS could explain the contribution of microalgae for greater shrimp weight gain. However, with the exception of astaxanthin, these nutrients were not limiting in the diet, thus

complicating this assessment. In the case of astaxanthin, Ju *et al.* (2009) demonstrated that adding the whole diatom *Thalassiosira weissflogi*, *Nannochloropsis* biomass or its residue fraction to a control diet significantly improve shrimp growth. This enhanced shrimp growth effect might not be derived from the macronutrient contribution of the algae biomass, and may be due instead to the presence of a growth factor (or factors) more likely present in the residue fraction of the algae biomass, rather than the acetone extract or carotenoid fraction, which did not effectively improve growth in that experiment. Consequently the improved growth of shrimp in the presence of microalgae was likely not due to astaxathin differences. The results of this trial are consistent with those of earlier work (Ju, Forster, Conquest & Dominy 2008), and indicated that phytoplankton in the floc culture had a major role in improving shrimp growth. Thus, the greater weight gain of shrimp in the GWS may be due to either 1) the continuous availability of microalgae nutrient(s) and greater concentration of energy, 2) greater digestion of unknown microalgae nutrients through the stimulation of the digestive enzymatic system of the shrimp (Moss *et al.* 2001; Divakaran & Moss 2004), 3) a growth hormone or insulin-like growth factor (Guillaume, Cruz-Ricque, Cuzon, Wormhoudt & Revol 1989), 4) substances that induce gene expression (Distel, Robinson & Spiegelman 1992; Fafournoux, Bruhat & Jousse 2000; Clarke, Gasperikova, Nelson, Lapillone & Heird 2002), 5) the phytoplankton contribution to a specific but unknown nutrient(s) in the shrimp diet, 6) and/or by affecting some water quality or other system factor. Further research is needed to investigate the causative agents in microalgae that promote shrimp growth.

Under these experimental conditions, this study demonstrated that in the presence and absence of microalgae, 4% dietary LT (3.5% PLs + 3.2% PUFAs) and 1% FO in addition to the other marine oils from squid and fish meals, increased juvenile *L. vannamei* growth compared to 1% dietary LT (1.5% PLs + 1.6% PUFAs), confirming that PLs together with LOA and LNA contained in soybean lecithin could reduce the quantitative requirements of HUFAs from FO in the white-legged shrimp. In addition, the combined effect of nutrients present in microalgae and/or specific growth factors could positively influence shrimp performance, which should be considered when formulating commercial diets and to optimize the phytoplankton cultures in the shrimp pond water.

CHAPTER V

SUMMARY AND CONCLUSIONS

Given sustainable management of wild fish stocks to produce fishmeal (FM) and fish oil (FO) for use in aquatic animal feeds, a key challenge is to grow the aquaculture industry within limits of available raw materials. This limited availability of FM and FO as ingredients for aquaculture feeds and the continuous growth of aquaculture impose strong pressure on the reduction of the dietary inclusion levels of these limited resources. At the same time, consumers and retailers have become increasingly interested in sustainability metrics, implicating that dietary inclusion levels of natural resources such as FM and FO need to be reduced. For these reasons, a series of experiments were conducted to evaluate the potential of phytoplankton to increase the availability of nutrients to shrimp and if dietary phospholipids (PLs) could increase the availability of essential fatty acids (EFAs), optimizing feedstuffs inclusion levels in commercial shrimp diets. Thus, weight gain, survival and feed conversion ratio (FCR) of *Litopenaeus vannamei* fed different dietary combinations of FM, squid meal (SM), FO and soybean lecithin (LT) in an indoor clear-water system and green-water system (presence of microalgae) were evaluated.

Findings from these feeding trials included determining a minimum combination of 6.5% FM and 5% SM in the presence of primary productivity together with the reduction of FO levels to 1% by adding 4% LT (3.5% PLs + 3.2% PUFAs) could contribute to reducing aquaculture's pressure on forage fisheries, and thus support a

more sustainable growth of commercial aquaculture. Indeed, the ratio of wild fisheries inputs to farmed shrimp outputs, or the "fish-in to fish-out ratio (FI/FO) for farmed shrimp based on results from the present study was 0.51 ((6.5% FM + 2% FO inclusion in diet / 24% yield of FM + 5% yield of FO from wild fish) x 1.74 FCR), meaning that half a kilogram of wild fish was needed to produce one kilogram of farmed shrimp obtained at their maximum growth and survival. These results could be rapidly applied to the aquafeed industry, as these trials have been done in the presence of primary productivity, the environment in which shrimp are typically cultured.

This study also demonstrated that with an adequate fertilization program in a reservoir tank, 60.7% Diatoms (52.7% *Chaetoceros* sp.) and 22.8% Cyanobacteria (22.6% *Oscillatoria* sp.) predominated, which contributes to a better management of intensive shrimp ponds with a normal predominance of 76.3% Cyanobacteria (54% *Oscillatoria*) observed for most of the 9 months in all 12 sampled ponds. These experimental results should be compared to commercial-scale systems, in order to determine the specific management of the fertilization program that will enhance diatom proliferation.

The contribution of phytoplankton to shrimp weight gain in the feeding trials of this study varied from 38.8 to 60.6%. This increased shrimp weight gain was not due to a specific nutrient, but to the combined effect of nutrients, and/or specific growth factors, which should be considered when formulating commercial diets.

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