การควบคุมคุณภาพน้ำในการเลี้ยงกุ้งโดยการเลี้ยงร่วมกับสาหร่ายสไปรูลินา *Spirulina platensis*

นางสาวเบ็ญจมาศ จันทะภา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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WATER QUALITY CONTROL IN SHRIMP CULTURE BY INTEGRATED CULTURE WITH THE MICROALGA, *Spirulina platensis*

Miss Benjamas Chuntapa

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By	Miss Benjamas Chuntapa
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Thesis Advisor	Professor Piamsak Menasveta, Ph.D.
Thesis Co-advisor	Sorawit Powtongsook, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctor's Degree

.....Dean of Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)

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(Sorawit Powtongsook, Ph.D.)

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(Associate Professor Somkiat Piyatiratitivorakul, Ph.D.)

.....Member
(Associate Professor Boosya Bunnag, M.Sc.)

เบ็ญจมาศ จันทะภา : การควบคุมคุณภาพน้ำในการเลี้ยงกุ้งโดยการเลี้ยงร่วมกับสาหร่ายสไปรูลินา *Spirulina platensis* (WATER QUALITY CONTROL IN SHRIMP CULTURE BY INTEGRATED CULTURE WITH THE MICROALGA, *Spirulina platensis*) อ. ที่ปรึกษา : ศ.ดร. เปี่ยมศักดิ์ เมนะเศวต, อ. ที่ปรึกษาร่วม : ดร. สรวิศ เผ่าทองศุข, 248 หน้า. ISBN 974-17-3036-5.

ในการศึกษาครั้งนี้ แบ่งการทดลองออกเป็น 3 การทดลอง คือ (1) ศึกษาจลนพลศาสตร์ของการนำธาตุแอมโมเนียม และไนเตรทเข้าสู่เซลล์สไปรูลินาในระดับความเค็ม 3 ระดับในห้องปฏิบัติการ (2) ศึกษาการเลี้ยงกุ้งร่วมกับสไปรูลินาในตู้กระจกเพื่อควบคุมคุณภาพน้ำ และ (3) การศึกษาประสิทธิภาพของสไปรูลินาในการบำบัดไนโตรเจนและฟอสฟอรัสในในถังเลี้ยงกุ้งขนาด 470 ลิตร ในสภาวะที่ได้รับแสงตามธรรมชาติ

ผลของความเค็มที่ระดับ 0, 15 และ 30 ส่วนในพันส่วน (psu) ต่อการนำธาตุอาหารแอมโมเนียมและไนเตรทเข้าสู่เซลล์ของสาหร่ายสไปรูลินาในสภาวะห้องปฏิบัติการแสดงให้เห็นว่า ที่ระดับความเค็มสูง 15 และ 30 psu สไปรูลินาจะมีการนำธาตุอาหารแอมโมเนียมและไนเตรทเข้าสู่เซลล์ได้ช้ากว่าที่ระดับความเค็ม 0 psu และผลจากการทดลองพบว่าค่าความเข้มข้นของสารอาหารที่ทำให้อัตราการนำเข้าเป็นครึ่งหนึ่งของอัตราสูงสุด (K_m) และอัตราการนำสารอาหารเข้าสู่เซลล์สูงสุด (V_{max}) ของแอมโมเนียมที่ระดับความเค็ม 0, 15 และ 30 psu มีค่า K_m เท่ากับ 5.470, 4.544, 3.717 mg NH_4-N L^{-1} และ V_{max} เท่ากับ 0.320, 0.200, 0.120 mg-NH_4-N mgChl-*a*$^{-1}$ h^{-1}, ตามลำดับ ในขณะที่ค่า K_m และ V_{max} ของไนเตรทที่ระดับความเค็ม 0, 15 และ 30 ส่วนในพันส่วน มีค่า K_m เท่ากับ 16.444, 5.342, 6.293 mg NO_3-N L^{-1} และค่า V_{max} เท่ากับ 0.732, 0.313, 0.206 mg-NO_3-N mgChl-*a*$^{-1}$ h^{-1}, ตามลำดับ

การเลี้ยงกุ้งกุลาดำวัยรุ่นร่วมกับสาหร่ายสไปรูลินาเพื่อควบคุมคุณภาพน้ำ ประกอบด้วยการทดลองย่อยสองครั้ง ครั้งแรกเป็นการศึกษาผลของการเก็บเกี่ยวสาหร่ายแบบกึ่งต่อเนื่องต่อคุณภาพน้ำในตู้กระจกเลี้ยงกุ้ง และการทดลองครั้งที่สองเป็นการศึกษาผลของความหนาแน่นของกุ้งสองระดับต่อการประสิทธิภาพการบำบัดคุณภาพน้ำโดยใช้สาหร่ายสไปรูลินา ผลการทดลองพบว่าคุณภาพน้ำในกลุ่มทดลอง (เลี้ยงกุ้งร่วมกับสาหร่าย) มีปริมาณความเข้มข้นของสารประกอบไนโตรเจน (NH_4-N, NO_2-N and NO_3-N) ต่ำกว่ากลุ่มควบคุม (ไม่มีสาหร่าย) อย่างมีนัยสำคัญ การเลี้ยงกุ้งร่วมกับสาหร่ายสไปรูลินาที่ระดับความหนาแน่นของกุ้ง 40 และ 80 ตัวต่อตารางเมตรของพื้นที่ก้นถัง (8 และ 15 ตัว/ตู้) ให้ผลเช่นเดียวกันกับการทดลองในครั้งแรก โดยการเก็บเกี่ยวสาหร่ายสไปรูลินาออกจะช่วยกำจัดสารประกอบไนโตรเจนออกจากน้ำและทำให้ไม่มีการสะสมของไนเตรทเกิดขึ้นในระบบทดลอง

การทดลองสุดท้ายเป็นการเลี้ยงกุ้งร่วมกับสไปรูลินาในสภาพกลางแจ้งในถังพลาสติกขนาด 480 ลิตร โดยแบ่งชุดทดลองออกเป็นชุดควบคุม (เลี้ยงกุ้งโดยไม่มีการเติมสไปรูลินา) ชุดทดลอง 1 (เลี้ยงกุ้งร่วมกับสาหร่ายสไปรูลินา) และชุดทดลอง 2 (เลี้ยงกุ้งร่วมกับสาหร่ายสไปรูลินาและมีโครงสร้างให้กุ้งเกาะอาศัย) ผลการทดลองพบว่าสารอาหารส่วนใหญ่ที่เข้าสู่ระบบทดลองมาจากอาหารกุ้ง ซึ่งคิดเป็นร้อยละ 51-53 ของไนโตรเจนและร้อยละ 56-60 ของฟอสฟอรัสทั้งหมด ส่วนสารอาหารในวันสุดท้ายของการทดลองส่วนใหญ่จะอยู่ในน้ำ คิดเป็นร้อยละ 6-37 ของไนโตรเจนและร้อยละ 30-35 ของฟอสฟอรัส เมื่อเทียบกับปริมาณสารอาหารที่เข้าสู่ระบบ การเก็บเกี่ยวสไปรูลินาจากระบบการเลี้ยงของชุดทดลอง 1 สามารถกำจัดไนโตรเจนออกได้ร้อยละ 4.8 และกำจัดฟอสฟอรัสออกได้ร้อยละ 8.3 นอกเหนือจากนั้นยังพบการเติบโตของสาหร่ายทะเลชนิดอื่นๆ ขึ้นปนเปื้อนในถังทดลองซึ่งสาหร่ายดังกล่าวสามารถกำจัดไนโตรเจนได้ร้อยละ 6-11 และกำจัดฟอสฟอรัสได้ร้อยละ 8 และโครงสร้างให้กุ้งเกาะอาศัยช่วยเพิ่มอัตราการรอดของกุ้งในชุดทดลองที่ 2 แต่ก็จะลดการเติบโตของสาหร่ายสไปรูลินาเนื่องจากโครงสร้างดังกล่าวไปบดบังแสงที่สาหร่ายต้องใช้ในการเติบโต

ภาควิชา วิทยาศาสตร์ทางทะเล	ลายมือชื่อนิสิต
สาขาวิชา วิทยาศาสตร์ทางทะเล	ลายมือชื่ออาจารย์ที่ปรึกษา
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BENJAMAS CHUNTAPA : WATER QUALITY CONTROL IN SHRIMP CULTURE BY INTEGRATED CULTURE WITH THE MICROALGA, *Spirulina platensis* THESIS ADVISOR : PROF. PIAMSAK MENASVETA, Ph.D. THESIS CO-ADVISOR : SORAWIT POWTONGSOOK, Ph.D.
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This study was an evaluation of using *Spirulina platensis* for water quality control in shrimp culture tanks. This was including the physiological study of *Spirulina* nutrient uptake kinetics at different salinity under laboratory condition, the use of semi-continuous harvesting to remove the excess algal biomass from the shrimp aquariums and the evaluation of nitrogen and phosphorus removal in the *Spirulina*-shrimp culture system under outdoor condition.

It was found that high salinity (15 and 30 psu) had strong affect to nitrogen uptake rate in both ammonium and nitrate forms but salinity seemed to have more affect to nitrate uptake than ammonium uptake. The half-saturation concentration (K_m) and maximum uptake rate (V_{max}) of ammonium uptake by *Spirulina* in various salinities (0, 15, and 30 psu) were 5.470, 4.544, 3.717 mg NH_4-N L^{-1} for K_m and 0.320, 0.200, 0.120 mg NH_4-N mg Chl-*a*$^{-1}$ $h^{-1}$for V_{max}, respectively. In addition, the K_m and V_{max} constants of nitrate uptake by *Spirulina* in various salinities (0, 15, and 30 psu) were 16.444, 5.342, 6.293 mg NO_3-N L^{-1} for K_m and 0.732, 0.313, 0.206 mg-NO_3-N mgChl-*a*$^{-1}$ h^{-1} for V_{max}, respectively.

In the second experiment, *S. platensis* was co-cultured with black tiger shrimp (*Penaeus monodon*) for water quality control. Two experimental trials were performed in order to evaluated the effects of: (1) three *Spirulina* trial conditions on inorganic nitrogen concentrations at one shrimp density. *Spirulina* trial conditions included: absent, non-harvested and semi-continuous harvesting, and (2) two shrimp densities on inorganic nitrogen concentrations, with and without *Spirulina*. The results showed that semi-continuous harvesting of *Spirulina* at one shrimp density resulted in significantly reduced ($P<0.05$) inorganic nitrogen concentrations (NH_4^+, NO_2^- and NO_3^-). With non-harvested *Spirulina*, considerable variability occurred with nitrogen concentrations. The factorial evaluation of shrimp density versus presence and absence of *Spirulina* resulted in greatly reduced nitrogenous compounds with *Spirulina* present regardless of shrimp density, and only moderately increased nitrogen with greater shrimp density. Without *Spirulina*, all nitrogen compounds were substantially elevated and shrimp survived was significantly reduced at high shrimp density.

The closed shrimp culture system in the last experiment consisted of three treatments *i.e.*, shrimp culture without *Spirulina* (control), shrimp culture with *Spirulina* harvest (treatment 1) and shrimp culture with *Spirulina* harvest and shelter (treatment 2) and was conducted in 480 L fiberglass tanks located outdoor. The results demonstrated that feed contributed about 51-53% nitrogen and 56-60% phosphorus of total nutrients input to the system. Major outputs of nutrients were accounted as dissolved in water fraction which ranged between 6-37% for nitrogen and 30-35% for phosphorus of the total inputs. Harvest of *Spirulina* in treatment 1 removed 4.8% of nitrogen and 8.3% of phosphorus from the culture system. Moreover, other sessile algae contaminated during the experiment also had a significant portion of nutrient removal from the tank, up to 6-11% of nitrogen and 8% of phosphorus removal. Shelter in the treatment 2 significantly increased survival rate of shrimp but decreased growth of *Spirulina* due to shading of the shelter.

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ปีการศึกษา 2545	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

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CHAPTER I

INTRODUCTION

Aquaculture has been rapidly developed in response to dramatic increase in seafood demand and decline in natural fisheries production due to pollution and over exploitation. In Thailand, black tiger shrimp (*Penaeus monodon*) is the most important aquacultural species in both production yield and market value. After 15 years of development, shrimp production in Thailand are among the most productive shrimp farm in the world (Fast and Menasveta, 2000). More than 109,695.6 tons of shrimp were exported between January to June 2000 (Shrimp culture newsletter, 2002). This made Thailand a leader in shrimp production.

Development in shrimp culture techniques, from extensive culture in large pond to intensive culture with strict feeding programme and sophisticated pond management, increase shrimp production from 0.05-0.5 tons/hectare/year (0.008-0.08 tons/Rai/year) in extensive culture to 5-20 tons/hectare/year (0.8-3.20 tons/Rai/year) in a typical intensive system (Fast, 1992a; Rosenberry, 1995). However, the main concern on production and profitability in 1990s is managing the system that becomes more environmental friendly. This concept is resulting in the introduction of the recirculating seawater systems and standard regulations by the Department of Fisheries especially a Good Aquaculture Practice (GAP) and a Code of Conduct (CoC) in shrimp farming (FAO, 1995; Barg *et al*., 1996; Marine Shrimp Culture Research Institute, Department of Fisheries, 2003).

In general, the success of a closed aquaculture recirculating system mainly depends on water quality management, especially nitrogen waste. Intensive shrimp culture uses 30-57 % protein feed with high feeding rates. Organic nitrogen waste from uneaten feed and shrimp excretions decomposes into toxic inorganic nitrogen compounds, including ammonia (NH_3 or NH_4^+) and nitrite (NO_2^-). With aerobic conditions, ammonia and nitrite are converted into relatively nontoxic nitrate (NO_3), but the high nitrate concentrations can stress shrimp. Water exchange is still recommended especially when nitrate concentration reach up to 50 mg NO_3-N L^{-1} (Hart and O'Sullivan, 1993). In earthen ponds, however, ammonia waste is assimilated and transferred through the food web and nitrogen cycle in the pond. Phytoplanktons (microalgae) therefore play a dominant role in nitrogen regulation and therefore stabilizing earthen pond water quality.

The idea of applying microalgae to take up nutrients has been tested in both lab and outdoor experiments but not yet in commercial scale. High rate algal pond that being used in industrial water treatment plant is one of the examples. Reviews of wastewater treatment using microalgae were documented (Oswald, 1988a; 1988b; 1991; Lincoln and Earle, 1990; De la Noue *et al*., 1992; Laliberte *et al*., 1994, Vonshak, 1997, Sanchez *et al*., 2001). Another modification is the use of immobilized algae in whatever entrapment or attachment conditions, but these techniques are expensive and not practical on large scales with aquaculture ponds (Brouers *et al*., 1989; Yang and Wang, 1990; Travieso *et al*., 1992; Mallick and Rai, 1994). There were also a few studies with

immobilized algal cells for water quality control in fish culture (Chen, 2001). The main disadvantage of using microalgae to control the water quality is that algal cells are not easily removed from the culture system. If algal cells are not removed, nitrogen compounds will be released back to the water after cell decomposition. Moreover, high microalgae concentrations can cause dissolved oxygen depletions during the night due to high respiration rate. Ideally, microalgae used with integrated aquaculture systems should have all of the following characteristics: [1] algal cells must be harvested by simple filtration; [2] easy to mass culture; [3] tolerate wide salinity range; and [4] algae are a valuable by product.

In this research, the cyanobacterium *Spirulina platensis* was chosen for nitrogen removal in shrimp culture pond because it meets all the above requirements. *Spirulina* cells appear as blue green multicellular filament composed of cylindrical cells arranged in helicoidal trichome. It is naturally found in several environments such as soil, marshes, freshwater, brackish water, seawater and even in thermal springs (Fogg *et al*., 1973; Carr and Whitten, 1982; Richmond, 1986). *Spirulina* is one of the most studied microalgal species. It grew well in seawater (5-30 psu salinity) and can be easily harvested using 22 microns plankton net. Moreover, *Spirulina* cells contain up to 60-70% protein (Ciferri, 1983) which can be used as feeding supplement in animals (Jassby, 1988 ; Shubert, 1988; Kay, 1991; Becker, 1988). Although *Spirulina* is one of the most widely studied microalgae, especially for wastewater treatment, most studies are with algal monoculture in high rate algal pond system (Tanticharoen *et al*., 1993; Phang *et al*., 2000), not with integrated systems containing both algae and an animal crop.

Plan of dissertation

The objectives of this study were to evaluate the possibility of using *Spirulina* for water quality control in shrimp culture tanks. This included including the physiological study of *Spirulina* nutrient uptake kinetics at different levels of salinity, the use of semi-continuous harvesting to remove the excess algal biomass from the tanks and the evaluation of nitrogen cycle in the *Spirulina*-shrimp integrated culture system. This study consisted of three experiments that were reported in three chapters.

The first experiment (Chapter III) involved the study on effect of three levels of salinity (0, 15 and 30 psu) on ammonium and nitrate uptake kinetics of *Spirulina* in laboratory conditions. The kinetics data was evaluated using Michaelis-Menten Equation.

The second experiment (Chapter IV) was the study on water quality in an integrated culture system consisting of shrimps with *Spirulina* in glass aquariums. In this experiment, semi-continuous harvesting technique was applied for regulating algal concentration in both high (83 shrimp m^{-2}) and low (44 shrimp m^{-2}) shrimp densities.

The last experiment (Chapter V) followed the second experiment but in larger scale (480 L outdoor tank) with nutrient budget assessment.

CHAPTER II

LITERATURE REVIEW

2.1 Morphology, ecology and life cycle of Spirulina

Spirulina is a multicellular, filamentous cyanobacteria. It is classified in Phylum Cyanophyta, Family Oscillatoriaceae. Under the microscope, *Spirulina* appears as blue green filaments with cylindrical cells arranged in unbranched, helicoidal trichomes without heterocysts (Richmond, 1986; Vonshak, 1997; **Fig. 2.1**). The filaments are motile, gliding along their axis. The helical shape of the trichome is the specific character of this genus, but the helical parameters (*i.e.*, pitch, length and helix dimensions) vary with species and even within the same species (**Fig. 2.2**). The cell diameter ranges from 1 to 3 μm in the smaller species and from 3 to 12 μm in the larger. According to Ciferri (1983) an authentic isolate of *S. platensis* from Shad and *S. maxima* from Mexico grown in the laboratory under identical conditions showed that *S. platensis* is characterized by a diameter of the helix of >35 to 50 μm and a pitch of 60 μm. For *S. maxima* these parameters were 50 to 60 and 80 μm, respectively.

Rich (1931) stated that *Spirulina platensis* and *S. maxima* were the typical dominant species in certain alkaline lakes in Africa and Mexico. Zarrouk (1966) described *Spirulina* as a thermophilic alga. The optimal temperature for its growth was between 35°C to 38°C. It was found abundance in warm, and shallow brackish lakes. *Spirulina* is an ubiquitous organism. Species of *Spirulina* are found in greatly different

environments such as in soil, freshwater, marshes, brackish water, seawater and even in extreme environment like thermal springs. Chiu *et al*. (1978) reported that about 30 species of *Spirulina* have been identified.

Figure 2.1 Photomicrograph of *Spirulina platensis*

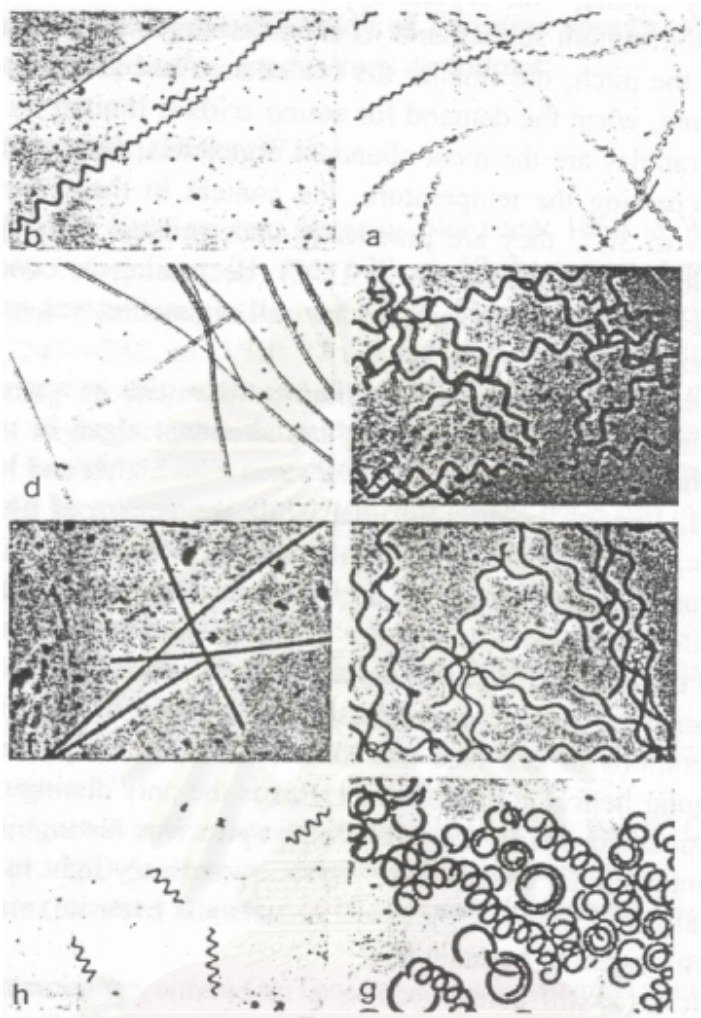

Figure 2.2 Different morphological types in *Spirulina* (Richmond, 1986): [a] Isolated from a local oxidation pond; [b] morphological similar trichome as in (a), developing from *Spirulina platensis* typical trichome; [c] *Spirulina platensis*, nonvacuolated from Lake Chad; [d] straight nonvacuolated trichomes, isolated from pure culture (c), from which they have been apparently transformed; [e] *Spirulina platensis*, vacuolated; [f] straight vacuolated trichomes, isolated from pure culture (b), (e), from which they have been apparently transformed; [g] *Spirulina sp*, apparently *platensis*, isolated from Lake Bogoria in Kenya; and [h] *Spirulina* (unidentified), gas vacuolated, appearing during the winter in a *Spirulina platensis* outdoor pond.

The life cycle of *Spirulina* in laboratory culture is rather simple (**Fig. 2.3**; Richmond, 1986). A mature trichome is broken in several pieces through the formation of specialized cells, necridia, that undergo lysis, giving biconcave separation disks. Trichome fragmentation located at necridia produces gliding, short chain (two to four cells) hormogonia, which is later a new trichome. The cells in a hormogonium lost the attached portions of the necridial cells, becoming rounded at the distal ends with little or no thickening of the walls. During this process, the cytoplasm appears less granulated and the cells assume a pale blue-green color. The number of cells in hormogonia increases by cell fission while the cytoplasm becomes granulated and the cells assume a brilliant blue-green colour. By this process trichomes increase in length and assume the typical helicoidal shape. Random but spontaneous breakage of trichomes together with the formation of necridia assure growth and dispersal of the organism.

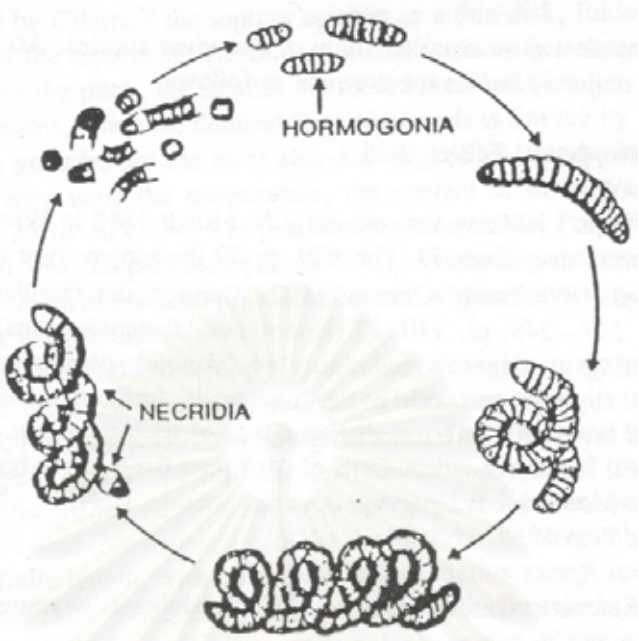

Figure 2.3 Life cycle of *Spirulina* (Richmond, 1986).

2.2 Factors affecting productivity of Spirulina

2.2.1 Nutrient requirement

2.2.1.1 Carbon source

All algae growing chemolithotrophically or photolithotrophically use dissolved CO_2 or one of its hydrated forms for the synthesis of organic compounds. In water, CO_2 may appear as H_2CO_3, HCO_3^-, or CO_3^{2-}, depending on the pH. In most natural freshwaters, the major pH buffer is the CO_2-H_2CO_3-HCO_3^--CO_3^{2-} system, which is also a very useful buffering system for mass culture of algae in high pH. In mass algal cultures, pH of the culture medium must be maintained at the optimum range for the

cultivated species and to prevent depletion of carbon. This is usually done by adding either CO_2 or $NaHCO_3$ in the culture medium (Kaplan *et al*., 1986).

The optimum pH for *Spirulina* growth is approximately 8.3 to 11. Alkalinity is essential for growth of *Spirulina*. *Spirulina* growth could quickly declined after a sudden change of pH. *Spirulina* culture medium containing 0.2 M $NaHCO_3$ therefore provides a good buffering capacity. Moreover, when alkalinity is low, the culture may become readily contaminated by other algal species (Richmond, 1986).

2.2.1.2. Nitrogen source

Various nitrogen compounds in either inorganic and organic forms can serve as nitrogen sources for the growth of microalgae. The ability to fix gaseous nitrogen is confined to prokaryotes, but the ability to use nitrate (NO_3^-), nitrite (NO_2^-), or ammonia (NH_4^+) appears to be general among algae where maximum growth rates being obtained with either NO_3^- or NH_4^+ (Richmond, 1986). After carbon (ignoring hydrogen and oxygen, which can be obtained from water), nitrogen is quantitatively the most important element contributing to the dry matter of algal cells. The proportion of nitrogen as the percent of dry weight can vary from 1 to 10%.

Nitrates are the main nitrogen source assimilated by *Spirulina*, but ammonium salt may be used as long as the NH_4^+ concentration is less than 100 mg nitrogen per litre (Richmond, 1988). At higher ammonium concentration, pH of the medium may fall

sharply, causing undesirable side effects. Urea can be used in *Spirulina* culture medium at pH 8.4, as long as its concentration is kept below approximately 1.5 g L^{-1}. Nitrite can also used as nitrogen source for *Spirulina* but only at low concentrations, approximately 1 mM. At higher concentration, nitrite inhibits growth (Morris, 1974).

2.2.1.3 Phosphorus source

Phosphorus is one of the major nutrient elements required for algal growth. It plays a major role in most cellular processes, particularly those involved in energy transfer and in nucleic acid synthesis. The major form in which microalgal cells acquire phosphorus is as inorganic phosphate ($H_2PO_4^-$ and HPO_4^{2-}). Phosphorus requirement for optimum growth differs considerably from species to species, even if no other external factors are limited. Uptake of phosphorus from the medium by algae is generally stimulated by light. Under phosphorus deficient condition, protein content, chlorophyll-*a*, RNA, DNA and cellular ATP content in eukaryotic and prokaryotic algal cells decreased, while carbohydrate content increased (Healey, 1982).

In addition to carbon, nitrogen, and phosphorus, other macro-nutrients required by algae are sulfur, calcium, magnesium, sodium, potassium, and chlorine. These nutrients are rarely deficient in algal growth media.

2.2.1.4 Microelements

In addition to macro-nutrients, micro-nutrients or microelements are required by *Spirulina* in relatively low quantity, with in the concentration range of mg L^{-1} to μgL^{-1}. The example of microelements needed for algal growth are iron, manganese, copper, molybdenum, vanadium, cobalt, nickel, silicon, selenium and vitamins.

2.2.2 Environmental factors

CO_2, temperature, and particularly light are among the major limiting factors for outdoor culture of algae. Generally, nutrients and CO_2 can be supplied to the culture at a reasonable cost. Light intensity and temperature are therefore the environmental factors that need to be seriously concerned, especially in an outdoor cultivation. Improper environmental condition therefore affect growth rate, photosynthesis and thus reduce productivity of the algal pond.

2.2.2.1 Light intensity

Light is the most important factor affecting photosynthetic organisms. *Spirulina*, like many other algae, grown photoautotrophically, depends on light as its main energy source (Vonshak, 1997). Due to the prokaryotic nature of *Spirulina*, light does not affect the differentiation or development processes. When nutrients and temperature are not limiting to growth, the most important environmental factor for mass

algal culture is light, the most effect of which concerns photosynthesis. Growth rate and biomass yield were regulated by the intensity and duration of the light irradiance. Light differs from other environmental factors because it is particularly available only at the surface of the culture. High light intensity leads to intense growth, produces relatively high O_2 evolution with a concomitantly low CO_2 in the pond. However, very strong light intensity condition possibly reduced growth rate as a result of photoinhibition.

Light limitation due to self-shading in dense culture is also affect microalgal growth. In an outdoor culture of *Spirulina*, Richmond and Grobbelaar (1986) illustrated that modification of the light regime by manipulating the depth of culture as well as cell density could reduce light limitation.

2.2.2.2 Temperature

While light is considered the most important environmental factor for photosynthetic organisms, temperature is undoubtedly the most fundamental factor for all living organisms. Temperature affects all metabolic activities including nutrient availability and uptake as well as other physical properties of the aqueous environment. Temperature is therefore a factor regulating algal growth and photosynthesis. In addition, metabolic regulation mechanism, structure of cell component (especially protein and lipid), specificity of enzyme reaction and cell membrane permeability are also temperature dependent.

The optimum temperature usually induced an exponential increase of the algal growth. When light intensity is constant and no nutritional limitation, maximum growth rate is function of temperature according to the Arrhenius equation (Vonshak, 1997). The decrease in the growth rate at high temperature may effect either a disruption of metabolic regulation or death of the cells. An Arrhenius plot for respiration showed an activation energy of 48.8 kJ mol^{-1} for *Spirulina*. The temperature coefficient (*Q*10) of the organism in a temperature range was calculated by the following equation, deduced from the Arrhenius equation (Pirt, 1975):

$$\log Q_e = \frac{E_a}{2.303R} \frac{10}{(T + 10)T}$$

E_a = the activation energy (kJ mol^{-1})

T = temperature

R = the universal gas constant (8.31 JK^{-1} mol^{-1})

Q_{10} of 1.85 was calculated for 20-40ºC. The respiration to photosynthesis ratio in *Spirulina* was 1 % at 20ºC and 4.6 % at 45ºC. These rather low values confirm the general assumption that cyanobacteria have low respiration rates (Van Liere and Mur, 1979). The respiration to photosynthesis rates measured in these experiments were much lower than those reported for outdoor cultures of *Spirulina*, where up to 34 per cent of the biomass produced during the daylight period may be lost through respiration at night (Guterman *et al*., 1989 cited in Vonshak, 1997; Torzillo *et al*., 1991a,b).

As many strains of *Spirulina* were originally isolated from temporal water bodies with a relatively high temperature. The usual optimal temperature for laboratory cultivation of *Spirulina* is in the range 35-38ºC. However, it must be pointed out that this range of temperature is arbitrary. Many *Spirulina* strains will differ in their optimal growth temperature, as well as their sensitivity to extreme ranges (Vonshak, 1997).

Torzillo *et al*. (1991a,b) studied the effect of temperature on productivity and night biomass loss of *Spirulina platensis* grown in outdoor photobioreactors with temperature control during May to September. They found that temperature had a significance influence on the productivity and biochemical composition of *Spirulina*. To obtain better biomass yield of *Spirulina*, it seems important to maintain the optimum temperature for growth during the entire light period, then reduce the temperature quickly with the onset of darkness. Moreover, it could be advantageous to harvest the daily production in the evening particularly when the rate of night biomass loss is expected to be high.

2.2.2.3 Mixing and Turbulence

Richmond and Becker (1986) suggested that in the mass culture of *Spirulina* without nutritional and environmental limiting to growth, then agitation to create a turbulent flow was the most important factor affecting the production yield. Torzillo (1997) also stated that mixing of the culture is needed by the three important reasons that are: (1) to ensure that all the cells are exposed to the light, (2) to maintain nutrient supply

and (3) to diminish the nutritional and gaseous gradient surrounding the cells in actively growing cultures. Stirring represents the most practical means by which efficient utilization of solar energy can be achieved (Richmond, 1990). When stirring is insufficient and flows pattern in the pond became laminar, the utilization of solar energy was then declined.

The reason of creating turbulent flow in the culture, however, relates to the phenomenon of mutual shading (Richmond, 1988). In high productive culture, illumination zone that sufficient for photosynthesis may be very shallow (15-30 cm). Turbulent flow induces a continuous movement relative position of the cells with respect to the photic zone, causing the solar radiation exposure at the pond surface more evenly to all cells in the culture (Richmond and Grobbelaar, 1986).

2.3 Application of Spirulina culture

2.3.1 Spirulina as for food

Use of *Spirulina* for health food aspect is due to its nutritional qualities such as high protein content (46-70% of dry weight; Becker, 1988), low fat, high vitamin content (particularly B12) and high in essential fatty acid especially gamma-linolenic acid (GLA C18:3) which seemed to have many therapeutic properties (Kunkel, 1982; Cohen *et al.*, 1987). Many works have been carried out using *Spirulina* as the additive in feed of several aquatic animals such as abalone, scallops, shrimp, and fish (Zhou *et al.*,

1991; Liao *et al*., 1993; El-Sayed, 1994; Mustafa *et al*., 1994). A specific application of *Spirulina*, mainly for colorant pigmentating agent in animals was also well studied.

2.3.2 Use of Spirulina for wastewater treatment

In exploring the role of algae in waste treatment, it should be noted that algal systems inevitably involve in wastewater treatment ponds of various types, the most common being facultative and high rate ponds. A facultative pond is usually more than a meter deep, has algae growing in its surface and is anoxic near the bottom. A high rate pond is usually less than a meter deep, is continuously mixed by gentle stirring and is aerobic throughout its volume.

Applying industrial and agricultural waste as a growth media for of *Spirulina* has been widely studied (Chung *et al*., 1978; Oron *et al*., 1979; Seshadri and Thomas, 1979; Tanticharoen *et al*., 1990; Fedler *et al*., 1993; Phang *et al*., 2000). The filamentous *Spirulina*, which may be separated from the medium with relative ease and has a high nutritive value is thus a promising organism for wastewater treatment.

Oron *et al*. (1979) grew *S. maxima* in raw cow manure waste in an outdoor pond, obtaining a yield of 3 g L^{-1}. An observation that may have useful potential was reported by Seshadri and Thomas (1979), who tested two species of *Spirulina* cultivated in outdoor pond, using low cost substitutes for some of the recommended nutrients. In particular, bone meal and biogas effluent were found to be very effective for *Spirulina*

growth. Integrating livestock waste recycling with production of microalgal, *Spirulina platensis* was studies by Fedler *et al* (1993). The results demonstrated that the maximum growth of *Spirulina* could be expected when the digested cattle waste was supplemented with 125% NO_3-N, 100% PO_4-P, 75-100% NH_3-N, and 2000-3000 mg L^{-1} HCO_3^-. Their conclusion strictly depended on the chemical composition of the digested cattle waste used. By keeping the same ratio among macro- and micro-nutrients and having the same ambient conditions, different digested cattle waste with different nutrient concentrations could be adjusted to obtain similar results. Decrease in light penetration into the culture and depleted concentrations of macro- or micro-nutrients were the major reasons for the reduction of growth.

The pilot scale cultivation of *Spirulina platensis* using tapioca starch wastewater from stabilization pond was studied in four high rate ponds (6x26x0.5 m^3) (Tanticharoen *et al*., 1990). The addition of sodium bicarbonate and nitrogen fertilizer raised the productivity up to 7-10 gram $m^{-2}day^{-1}$. The technology is already transferred to the private sector in which the factory was being built to produce 36 Tons dry *Spirulina* a year as animal feed.

2.4 Nutrient uptake kinetics research in algae

2.4.1 Measurement of algal nutrient uptake rate

The rate in which phytoplankton take up nutrients depends on their nutritional history. When phytoplankton are growing under nutrient saturated conditions, the uptake rate is equal to growth rate when both are expressed in units of time. But as cells become nutrient-starved or limiting, the potential uptake rate and growth rate become uncoupled. When these nutrient-limited cells are exposed to an addition of the limiting nutrient, their maximal nutrient uptake rate can be up to several orders of magnitude higher than the maximal growth rate (Harrison, 1988).

The general nutrient uptake rate evaluation method of algae and seaweed was described by Lobban and Harrison (1994). Uptake rate can be determined directly by using isotopes such as ^{32}P or indirectly by measuring the disappearance of the nutrient from the medium. The relationship between uptake rate of ions and its external concentration can be described by a rectangular hyperbola curve. Among various kinetics equations, Michaelis-Menten equation for enzyme kinetics has been the most accepted for nutrients uptake kinetics study in many organisms. The equation is described as:

$$V = \frac{V_{max} \cdot S}{K_m + S}$$

Definition:

V = nutrient uptake rate

(amount of nutrient per unit mass of algae per time *e.g.* mg-N g-algae^{-1} h^{-1})

V_{max} = the maximum uptake rate

(amount of nutrient per unit mass of algae per time)

K_m = the half-saturation constant

(amount of nutrient per unit volume at half of V_{max} *e.g.*, mg-N L^{-1})

S = the concentration of the limiting nutrient

(amount of nutrient per unit volume)

However, since the Michaelis-Menten equation is non-linear, data transformation is then required. Several transformation equations have been proposed in which K_m and V_{max} can be calculated more accurately by linear regression. However, the most accepted method for linear transformation, especially in nutrients uptake kinetics, is through Hanes-Woolf plot (Kopozak, 1994; Qi and Zhu, 1994; Taylor and Rees, 1999). The maximum nutrient uptake rate defined as V_{max} and the half-saturation constant defined as K_m were therefore calculated and used to fit the kinetics equation. Example of Hanes-Woolf plot is showed in **Fig. 2.4**.

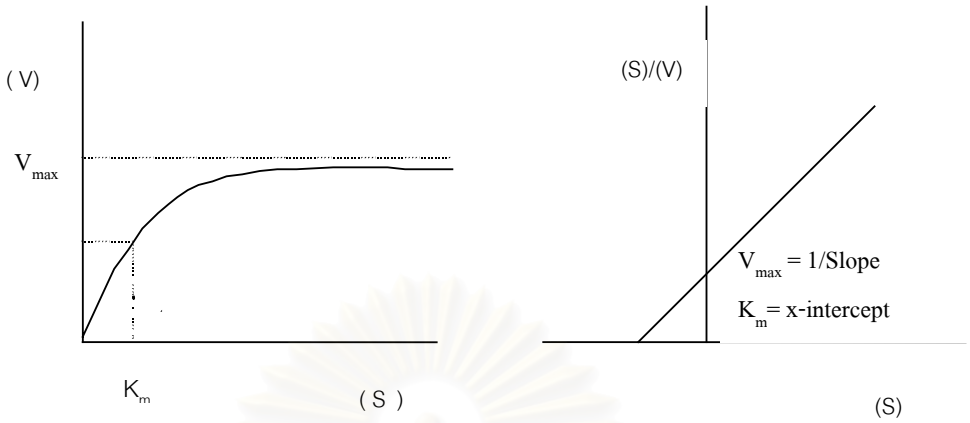

Figure 2.4 [a] The relationship between nutrient uptake rate (V) and nutrient concentration (S) according to Michaelis-Menten equation kinetic model; [b] Hanes-Woolf's plot between S/V and S is used for evaluation of V_{max} and $K_{m.}$

After Hanes-Woolf tranformation, slope of the plot of S/V versus S is equal to $1/V_{max}$ and the linear regression line is across X axis at $-K_m$ value. By this transformation, V_{max} and K_m can be calculated from the regression equation.

The lower the value of K_m, the higher the affinity of the carrier site for the particular ion. The transport capabilities of a particular phytoplankton are generally described by parameters V_{max} and K_m. It is important to realize that these parameters do not have a constant value for each species. They vary as a result of nutritional past history, light, temperature and other nutrient interactions. Healey (1980) suggested that the slope of the initial part of the hyperbola (the linear portion) may be a more useful

parameter for comparing the competitive ability of various species for a limiting nutrient; which is similar in concept to the use of the slope.

2.4.2 Algal nutrient uptake rate

The K_m values of seaweed are generally high (2-40 µM), up to 10 times higher than phytoplankton, and the V_{max} values range from 3 to 188 umol g_{dw}^{-1} h^{-1} for both nitrate and ammonium. Because specific uptake rates (where the rate is normalized to the nitrogen content of the thallus) generally are not reported for seaweed, comparisons with phytoplankton are seldom possible (Lobban and Harrison, 1994). Therefore, V_{max} values for ammonium measured over 0-10 minutes may be much higher than values obtained over 0-60 minutes, because uptake rates decrease with time as the intracellular pools of nitrogen limited algae fill. In contrast, nitrate uptake by nitrogen limited algae may increase over 1-2 h, as added nitrate induced more rapid uptake (Thomas and Harrison, 1987). Preliminary studies of Lobban and Harrison (1994) indicated that nitrogen assimilation (the incorporation of NO_3^- and $NH4^+$ into amino acids and proteins) in seaweeds is similar to that in phytoplankton, although these processes in phytoplankton usually are faster. Since no macroalgae are known to fix N_2 (except those containing symbionts) the inorganic sources for these plants are nitrate, nitrite and ammonium.

Dugdale (1967) suggested the hypothetical examples of interspecies competition based upon assumed values of K_m and V_{max}. Eppley *et al.* (1969) showed

that competition between *Skeletonema costatum* and *Euriliania huxleyi* could be accounted by their difference in K_m for nitrate and ammonium. Furthermore, Titman (1976) also reported the resource-based competition theory with K_m for different species and nutrients. Lehman *et al*. (1975) reported that average V_{max} values for diatoms and dinoflagellates was approximately 20 and 100 ($x10^{-9}$ umoles.cell^{-1}.h^{-1}), respectively. The ability of nitrate utilization by an organism in nutrient deficient seawater is expressed well by these two parameters (Morel, 1987).

A comparative study of nitrate uptake has been undertaken with nitrogen-deficient cells of the dinoflagellate, *Prorocentrum micans* and the diatom, *Chaetoceros lorenzianus* (Qi and Zhu, 1994). Uptake rates appeared to be hyperbolic with nitrate concentration between 0.2 to 8.0 µM. The maximum uptake rates (V_{max}) and K_m showed measurable difference between the two species as V_{max} of *P. micans* and *C. lorenzianus* were 4.72 and 0.48 ($x10^{-6}$ µM.h^{-1}.cell^{-1}), respectively and K_m were 1.55 and 4.50 (µM), respectively. Since *P. micans* had higher V_{max} and lower K_m than *C. lorenzianus*. Thus, competition between the dinoflagellate *P. micans* and the diatom *C. lorenzianus* was a result of different nutrient uptake rate where *P. micans* which had lower K_m was become a dominant species in most situation. On the other hand, *C. lorenzianus* that had higher K_m became competitively later.

Ammonium and nitrate uptakes were measured in juvenile *Laminaria saccharina* and *Nereocystis luetkeana* originating from a salmon sea cage farm in northwestern British Columbia, Canada (Ahn *et al*., 1998). The effect of various

concentrations of NH_4^+ and NO_3^-, within the range that usually found around the typical salmon farm environment on nitrogen uptake rate was examined. They reported the ammonium uptake rate of 6.0-8.9 and 6.6-9.3 $\mu M.g_{dw}^{-1}.h^{-1}$ and nitrate uptake rate of 4.6-10.6 and 6.1-17.0 $\mu M.g_{dw}^{-1}.h^{-1}$ for *L. saccharina* and *N. luetkeana*, respectively.

There was very few study in nitrite uptake of algae. In *Codium fragile*, the maximum uptake rate for nitrite was found to be similar to nitrate, but lower than ammonium (Hanisak and Harlin, 1978). However, Brinkhuis *et al*. (1989) observed a rapid nitrite uptake lasting only a few minutes, followed by a release of some of that nitrite back to the medium, then the uptake rate was sustained for several hours.

Nitrogen uptake rates by macroalgae depended on the concentration of nitrogen sources, and on environmental factors such as light, temperature, and water movements (Haines and Wheeler, 1978; Lobban and Harrison, 1994). Ahn *et al*. (1998) suggested that the uptake rate of nutrients is influenced by several biological factors such as, the various types of tissue, the age of the plant, its nutritional past history or nitrogen status of the thallus (in macroalgae). Genetics as well as physiological factors had been used to explain the variation in uptake rates under different environmental levels of nitrogen. Even the same species of phytoplankton isolated from different location of various nutrient availability could have different nutrient-uptake kinetics (Carpenter and Guillard, 1971).

The feasibility of biological nitrate removal from groundwater was performed using cyanobacterial cultures under laboratory conditions (Hu *et al*,. 2000). Their results demonstrated that when cyanobacteria were grown, nitrate was continuously removed from the water. All three cyanobacterial species (*Synehocystis* sp. strain PCC 6803, *Synchocystis minima* CCAP 1480, and *Synechococcus* sp. strain PCC 7942) showed rapid removal of nitrate from groundwater with the average nitrate uptake rate up to 0.05 mM $NO_3^-.h^{-1}$.

2.5 Transformation of nitrogen compound in aquatic environment

Elemental nitrogen (N_2), accounted 78% of the atmosphere, is very inactive because of the strong bond (three electron pairs) between the nitrogen atoms. Some

bacteria (associated with certain plants) and certain blue-green algae are able to fix nitrogen and assimilate it as organic nitrogen. Spotte (1979) summarized the nitrogen cycle with four possible ways by which nitrogen enters water: 1) diffusion from the atmosphere or from subsurface air bubble; 2) excretion by macroalgae; 3) excretion by metazoan animal and 4) oxidation processes of heterotrophic bacteria. The nirogen cycle in water is shown in **Fig. 2.5**.

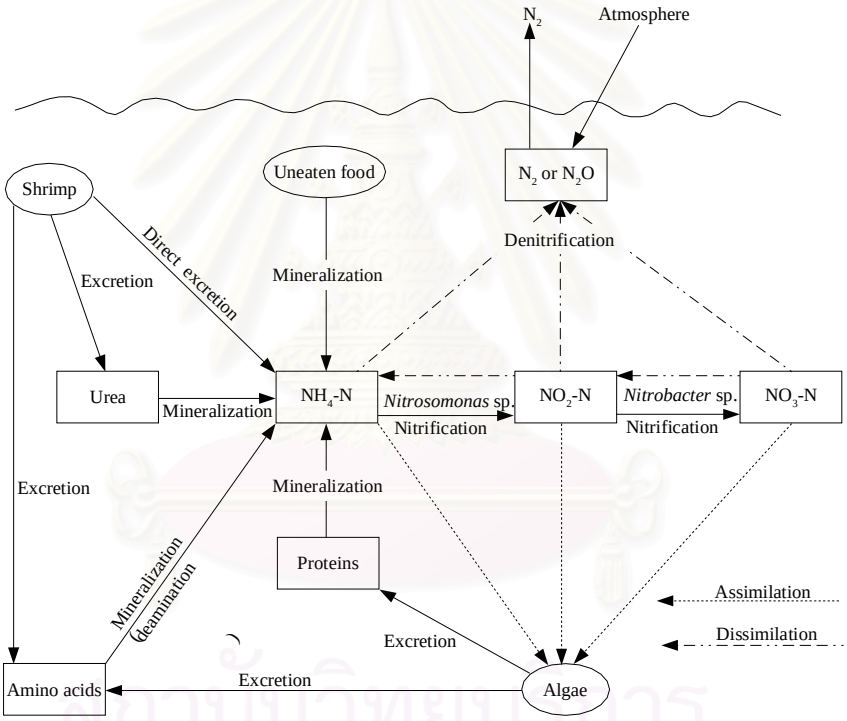

Figure 2.5 The nitrogen cycle in water (Modified from Spotte, 1979)

The first step of nitrogen transformation, decomposition of organic matter (dead organisms and uneaten food) by microbes transforms organic nitrogen to ammonia (NH_3) or ammonium (NH_4^+). This process is known as mineralization of organic nitrogen. When organic nitrogen is mineralized to inorganic nitrogen or ammonium

fertilizer is applied, a pH-temperature dependent equilibrium is established between NH_3 and NH_4^+:

$$NH_3 + H^+ \leftrightarrow NH_4^+$$

The second step of N-transformation is the two-stages aerobic process of nitrification. The first-stage of nitrification is the conversion of ammonia or ammonium to nitrite by chemoautotrophic bacteria such as *Nitrosomonas*:

$$NH_4^+ + 1_{1/2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

The second-stage of nitrification process, oxidation of nitrite to nitrate, is then accomplished by another chemoautotrophic bacteria especially the genus *Nitrobacter*:

$$NO_2^- + {}_{1/2}O_2 \rightarrow NO_3^-$$

The finally process of nitrogen-transformation is denitrification. Nitrate is soluble and easily lost through leaching. Nitrate also can be lost through denitrification. Boyd (1995) summarized the pathway of denitrification in several steps that are:

$$HNO_3 + 2H^+ \rightarrow HNO_2 + H_2O$$

$$2HNO_2 + 4H^+ \rightarrow H_2N_2O_2 \text{ (hyponitrite)} + 2H_2O$$

$$H_2N_2O_2 + 4H^+ \rightarrow 2HN_2OH \text{ (hydroxylamine)}$$

$$NH_2OH + 2H^+ \rightarrow NH_3 + 2H_2O$$

$$H_2N_2O_2 + 2H^+ \rightarrow N_2O + H_2O$$

$$H_2N_2O_2 + 2H^+ \rightarrow N_2 + 2H_2O$$

$$N_2O \text{ (nitrous oxide)} + 2H^+ \rightarrow N_2 + H_2O$$

Denitrification occurs under strictly anaerobic condition. Microorganisms that carry out these transformations use oxidized inorganic compounds (*e.g.*, NO_3^-, NO_2^-) as terminal electron and proton acceptors instead of molecular oxygen in respiration.

2.6 Transformation of phosphorus compound in aquatic environment

Phosphorus exists in water in three states: dissolved inorganic phosphorus (DIP, also called phosphate, orthophosphate, and reactive phosphate), dissolved organic phosphorus (DOP), and particulate organic phosphorus (POP). The last form is associated with floating detritus particles and the cells of suspended microorganisms. Orthophosphate is the most important form in nature, and the same is probably true in water. In seawater of pH 8, 1% of the orthophosphate is present as $H_2PO_4^-$, 87% as HPO_4^{2-}, and 12% as PO_4^{2-} (Kester and Pytkowicz, 1967).

In nature, the enormous mass of the primary producers (phytoplankton, in particular) and primary and secondary consumers (herbivorous and carnivorous zooplankton) account for most of the phosphorus cycling. The onset of a phytoplankton bloom triggers a rise in all forms of phosphorus. On the other hand, the role of planktonic organisms in the phosphorus cycle in water is insignificant. Phosphorus enters water in four ways: (1) excretion by algae; (2) excretion by metazoan animals; (3) autolysis and release from damaged cells; and (4) lysis of dead cell and mineralization of dissolved and particulate organic matter by heterotrophic bacteria (Spotte, 1979).

The phosphorus cycle in water is illustrated in **Fig. 2.6**. Macroalgae excrete DOP. A portion is reused during growth, whereas the rest is mineralized to the inorganic state (DIP) by heterotrophic bacteria. In addition, macroalgae excrete a certain amount of DIP. Both DOP and DIP are reassimilated by macroalgae. According to Owens and Esaias (1976), no evidence exists that algae can directly assimilate forms of phosphrus other than orthophosphate; however DOP can be hydrolyzed near the outer cell membranes and utilized indirectly.

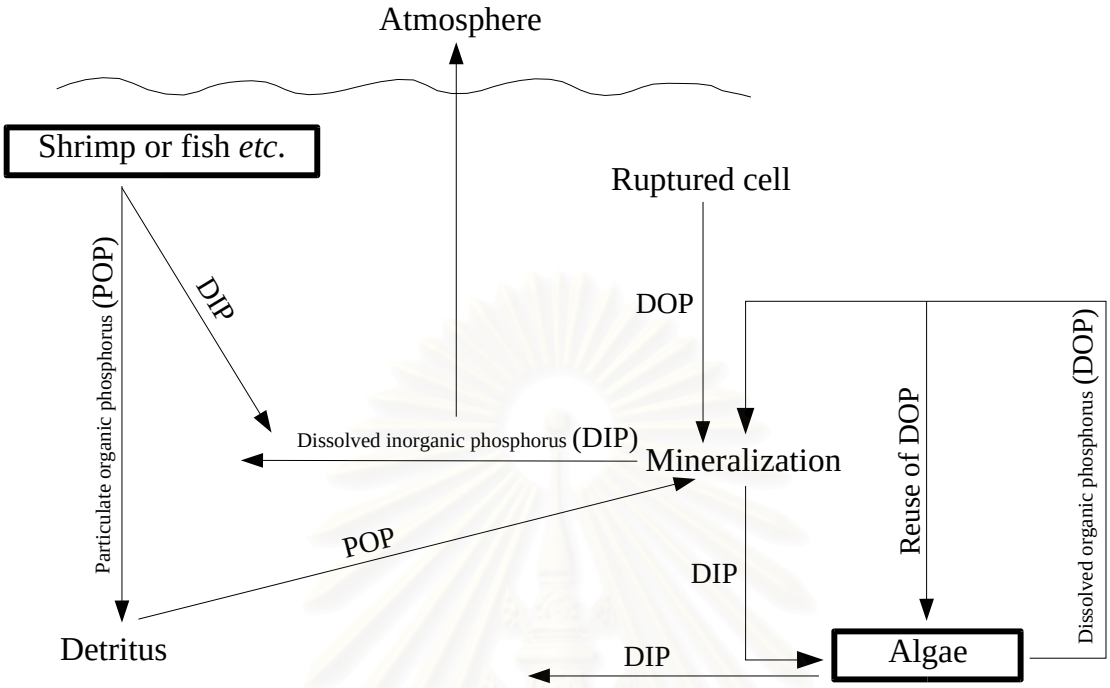

Figure 2.6 Phosphorus cycle of water (Spotte, 1979).

Ruptured cells of animals, bacteria, and algae release DOP, some of which is mineralized by heterotrophic bacteria. Metazoan animals excrete some of their phosphorus in the inorganic state and the remainder appears in fecal material as POP. Some of the phosphorus in animal feces sinks to the bottom of the aquarium and is adsorbed onto detritus. When part of the detritus is mineralized, inorganic phosphorus is liberated. Excess inorganic phosphorus from the excretion of metazoan animals and macroalgae, and from mineralization of organic phosphorus by heterotrophic bacteria, appears collectively as reactive phosphate when measured in the laboratory.

2.7 Biological nutrient treatment methods

The term of biological filtration in the broad sense refers to filtration techniques that use living organisms to remove a substance from a liquid solution. This definition includes systems that use bacteria, algae and higher green plants to filter the water. These plant systems are commonly referred as the hydroponic systems. The term of biological filter, or biofilter, as used here also refers to the remove of ammonia and nitrite by microalgae.

For shrimp farms, fish and/or mollusks can be stocked in treatment ponds or effluent canals. Fish and mollusks play important roles in nutrient recycling, converting living or dead organic particles into inorganic nutrients, which in turn stimulate a phytoplankton bloom, called “icthyo-eutrophication”. The species used for biological treatment on shrimp farms are detritus and/or plankton feeding fish (*e.g*. mullet and milkfisk) and mollusks (*e.g*., mussels and oysters) (Lin, 1995).

2.8 Recirculation water system in aquaculture

The traditional method for removing wastes from aquaculture ponds has been by frequent water exchange. To reduce the waste discharged from shrimp pond to the environment, closed water recycling systems were recently developed (Lin, 1993). The carrying capacity of the water recycling system is based on the balance between the quantity of waste products being generated by shrimp and the capacity to assimilate those

wastes by microorganisms. The general scheme is similar to a simplified version of domestic waste treatment, which includes sedimentation ponds, biological treatment and aeration (Timmons and Losordo, 1994). Finally, the treated water is stored in a reservoir pond and returned to shrimp growout pond.

Recently, complete recirculation systems were used mostly for experiment culture applications involving relatively small water volumes, or for relatively small commercial ventures involving high value crops such as ornamental fish (Adey and Loveland, 1991), fish and crustacean seed for conventional growout or stock enhancement (Menasveta *et al*., 1989, 1991, 2001; Tseng *et al*., 1998), or for research (Yang *et al*., 1989). Fast (1992a,b) described many innovations in pond growout techniques that occurred during the 1980's and earlier. During the past, recirculation systems have been applied commercially to marine shrimp growout on much larger scales than previously envisioned. However, rigorous research on pond dynamics and engineering approach to design is needed to optimize the culture systems (Lin, 1995).

Recirculation systems for intensive shrimp culture were initially introduced into the problematic shrimp farming areas in central and eastern Thailand a few years ago and have been widely used by many farmers in Thailand and Indonesia with considerable success. Research based on a similar concept of "zero water discharge" for shrimp culture has been also conducted in South Carolina, USA (Hopkins *et al*., 1995) and for fish in Israel (Avnimelech *et al*., 1987; Krom and Neori, 1989). While the concept of

water recycling systems is ecologically sound, the efficiency of the system is still far from being perfected.

2.9 Nutrient bioremoval from aquaculture ponds

Many attempts to remove nutrients waste from aquaculture using biological activities have been examined for years. One of the examples is the use of filter feeder such as oyster to remove the excess amount of phytoplankton and suspended detritus in shrimp ponds. This included an integration of oyster with seaweed such as *Gracilaria* or *Caulerpa* which was introduced by the Department of Fisheries in Thailand.

One of the shellfish species used in aquaculture ponds is mussel (*Mytilus* spp.). Like other bivalves, mussel is a filter feeder that grew well in extensive range of environmental conditions. In general, filtering activity of mussels is related with strains, water temperature and the amount of phytoplankton available. Generally, the filtration rate is inversely proportional to the concentration of algal cells in the water. For example, at an algal cells concentration of $5x10^5$ cells L^{-1}, the mussels filtration rate was 3 liters/hour/mussel, while at $2x10^7$ algal cells L^{-1} the filtration rate was reduced to 0.5 liters/hour/mussel (Landau, 1992).

In an integrated oyster and shrimp system, phytoplankton production is greatly stimulated by the continuous harvesting of phytoplankton by the oysters. Wang and Jakob (1991) illustrated that the reduction of phytoplankton density in the water during night provided a positive effect on the dissolved oxygen concentration in the pond.

The stimulation of phytoplankton growth, as a result of continuous filtering by oysters, increased the growth of phytoplankton that helped in the regulation of pH, ammonia and nitrite concentrations in the pond. Imai (1971) cited in Weston (1991) showed that 23% of the organic particles including phytoplankton could be removed by a single oyster raft and eventually reduced by 94% after flow through 11 rafts of oyster. It was also noted that the consumption of phytoplankton by cultured bivalves species could possibly reduced the food supply available to zooplankton in the pond (Incze and Lutz, 1980; Rosenberg and Loo, 1983; Rodhous and Roden, 1987).

The University of Hawaii Integrated Recirculating Oyster/Shrimp Production Systems is based upon a simple concept (Wang, 1998). In an oyster/shrimp system, the excess nutrients in the shrimp tank can be used to produce a marine microalgal such as *Chaetoceros* sp. The algal water is then pumped to a fluidized packed oyster column where oysters are suspended individually in a stream of high velocity water from the shrimp tank. The oyster feed on the algae, thereby eliminating oyster food cost while reducing the excessive nutrient load caused by the incomplete utilization of the shrimp feed. After the algae have been removed, the water was then return to the shrimp tank to be reused. Up to 95% of sustained water reuse has been achieved. The normal range of water reuse is 80% to 90%. The system rests on two patents: The first is the Fluidized Bed technology, and the second is a pending patent application on the controlled production of marine algae in an open system. The ability to control the algal species is important to the success of the system, since it must be the right food for the oysters. By controlling the nutrient input to the tank, and by making sure there are sufficient oysters

to remove algae continuously, a desired dominate algal species in the system can be maintained. A venture capital group has licensed the patents from the University of Hawaii and created the Kona Bay Oyster & Shrimp Company in 1997. The company has begun its operation and is expected to reach full production in late 1998. Flat-bottom twenty-eight foot diameters round shrimp tanks with center drains are used. Circular water motion is maintained in the tanks to remove all settleable solid. The oyster columns, 18 inches in diameter and 6 feet in height, can contain about 3,000 of 55 gram size American Cup oysters (Wang, 1998).

2.10 Sources of nitrogen and phosphorus in shrimp pond

Waste in the intensive aquaculture pond is predominantly from undigested feed residues and excretion products (Cripps, 1993). Feed pellet stability, nutritional formulation, and feeding practice greatly influence the total waste loading. Hence, intensive fish farming therefore generates large amounts of both particulate organic nitrogen waste (uneaten food and feces) and dissolved excretory products due to inefficient use of the diet, up to 25% of total nitrogen waste (Handy and Poxton, 1983; Porter *et al*., 1987; Dosdat *et al*., 1996). Thus, dissolved nitrogen compounds play an important role in aquaculture water quality, since they can be nutrients for primary production, as well as potential fish toxicants (Lorenzen *et al*., 1997).

2.11 Water quality in intensive shrimp culture

Chen *et al.* (1989) reported that production yield of *Penaeus monodon* in Taiwan varied from 2.7 to 21 tons ha^{-1} with a stocking density of 21-143 PL m^{-2} and FCR 1.39 to 2.33. Funge-Smith and Briggs (1998) reported that intensive shrimp culture in Thailand involved relatively high stocking densities (50-100 m^{-2}), high production (6-12 Tons ha^{-1} $crop^{-1}$), high feeding rate (with FCR 1.8-2.0) and high water exchange rate (up to 5-10% per day towards harvest time). In general, intensive shrimp farming requires high feeding rates due to high stocking density (Briggs and Funge-Smith, 1994). Most nutritional supplement is from artificial feed with strictly feeding programs (Fast and Lannan, 1992). Briggs and Funge-Smith (1994) found that nutrient concentrations were invariably higher in the pond with higher stocking density than the pond with lower stocking density. The absolute protein requirement of juvenile shrimp was up to 35 - 40 % crude protein (Alava and Lim, 1983; Akiyama *et al.*, 1992). Increasing protein levels in feed is not only discouraging in economic sense, but it also has adverse effects on water quality.

Various feed compositions are applied in fish and shrimp farming in order to increase production. Feed conversion ratio (FCR) of black tiger shrimp fed with commercial formulated feed commonly ranged from 1.5 to 1.8 in Taiwan and the Philippines (Liu and Mancebo, 1983; Chen *et al*., 1989). Muthuwan (1991) reported FCR of 1.7 in commercial farming in Thailand. However, in intensive shrimp culture, FCR between 2 to 3 was possibly found (Boyd and Watten, 1989). One of the reasons of high conversion ratio is overfeeding.

In intensive shrimp culture with high stocking density and high feeding rate consequently produced large amount of metabolic waste. Ponds became more eutrophic with rapid deterioration of water quality (Mevel and Chamroux, 1981). Basically, vast amount of feed which is not fully utilized by fish thus creating a large quantity of available nutrients in the water column and at the bottom sediment (Zur, 1981). Respiration of dense fish population continuously supply carbon dioxide in to the pond and usually promote phytoplankton growth or even cause dangerous algal bloom, which can lead to fish mortality (Zur, 1981). In addition, respiration of plankton and bacteria influences an extremely low oxygen concentration during the night and early morning.

Dissolved oxygen (DO) is one of the most important water quality parameter in aquaculture (Boyd and Watten, 1989). Critically low DO levels in the pond, particularly after an algal bloom (Chang and Quyang, 1988), can cause stress or even mortality of shrimp (Madenjian *et al.*, 1987). Emergency aeration can often prevent mortality (Boyd and Watten, 1989), however, chronically low DO can reduce growth, feed consumption and moulting frequency (Clark, 1986; Chang and Ouyang, 1988). Allan and Maguire (1991) reported that the lethal DO level for *P. monodon* was between 0.5 and 1.0 mg L^{-1}. They also suggested that *P. monodon* are very resilient in reponse to a short-term DO stress. Moreover, an increase in ammonia concentration and decrease in pH often accompany rapid DO declines (Boyd, 1982). Thus it is critical that the supplemental aeration device is essential for intensive production ponds. Insufficient aeration may result in critically low DO and mass mortality. Very high aeration, on the other hand, can increase erosion the pond. Optimum aeration usually depends on factors

like feeding rate, standing biomass of shrimp and phytoplankton concentration as well as environmental condition.

Organic loading in shrimp pond is a result mainly from the residual feed, shrimp faeces and metabolic products of shrimp. The amount of organic loading is directly related to stocking density and culture duration. Data compiled by Boyd (1992) showed that the feeding rate went up to 400 kg ha^{-1} day^{-1} at a stocking density of 50 PL m^{-2}. The accumulated organic matter was decomposed by either aerobic and anaerobic processes. Boyd (1992) reviewed that the factors affecting organic matter decomposition were composition of organic matter, temperature, pH, nutrient supply and oxygen supply.

The major concern of water quality in the closed system is the accumulation of ammonia and nitrite in the culture system. Ammonia is toxic to aquatic animals and cause impairment of cerebral energy metabolism and damage to gill, liver, kidney and thyroid tissue in fish, crustaceans and molluscs (Allan *et al*., 1990). It can limit production yield of intensive fish and crustacean aquaculture (Allan *et al*., 1990) and even cause mass mortality of fish in natural condition (Tarzona *et al*., 1987). Ammonia is the principal end product of nitrogenous compound in an ammonification of organic matter and deamination of aquatic animal excretion (Chen and Tu, 1991). Ammonia conversion is then occur as nitrification process which is carried out in two steps, conversion of ammonia to nitrite by *Nitrosomonas* followed by further conversion of nitrite to nitrate by *Nitrobactor*. In case of high amount of waste released into the pond

by cultured animals, the bacterial flora occasionally can not respond rapidly enough to prevent a built up of nitrite at the bottom.

Ammonia in culture system may persist in two forms *i.e.* ionized and unionized forms, in which the latter is more toxic to aquatic animals. The ionization of ammonia depends on temperature, salinity and pH of water (Wickins, 1976). Chen and Tu (1991) found that ammonia and nitrite concentration in the intensive culture frequently rise to the level that can cause stress to shrimp. In growout pond of *P. monodon*, the total ammonia may as high as to 6.5 mg L^{-1} even with frequent water exchange. Chiayvareesajja and Boyd (1993) reported that the only feasible means of reducing ammonia concentration in the culture system was water exchange. However, the green water with active phytoplankton are capable to take up ammonia (Moll, 1986). The shrimp biomass still under carrying capacity of the culture system (closed system), whose waste was removed by phytoplankton and some micro-organisms (Keawchum, 1994).

Chen *et al*. (1990) reported that LC_{50} at 24, 48 and 72 h of ammonia for *P. monodon* adolescent were 1.76, 1.59 and 1.20 mg L^{-1} of NH_3-N respectively. While the LC_{50} of nitrite at 24, 48, and 72 h were 218, 193 and 185 mg L^{-1}, respectively. They found that low DO caused a reduction in the acutely lethal levels of ammonia for prawn down to 0.5 mg NH_3-N L^{-1}.

Recently, biotechnology which involves the manipulation of micro-organisms to enhance mineralization of organic matter and get rid of undersirable waste compound has been exploited in many aspects. In the field of aquaculture, adding bacteria or enzyme in the pond has been made to improve water quality especially by increase the decomposition rate in the pond. Boyd (1990) concluded that commercial suspension of bacteria was ineffective and in fact those products only contained common saprophytic bacteria. Those bacteria were common in aquaculture environment and normally in abundance if supplied with appropriate substrate *e.g.* carbon and nitrogen compounds. The key of water quality management is to continuously resuspension of organic particle under aerobic condition in order to increase oxidation and microbial activity. Schroeder (1978) also emphasized that adequate nutrient were needed to achieve rapid and efficient activity by micro-organisms. It is not necessary to add bacteria since they are already present. Keawchum (1994) also found that the application of bacteria did not give any benefit to the shrimp growth and water quality in the culture system.

Most studied in nutrient treatment in aquaculture pond have been focused on the removal or conversion of nitrogenous compounds to other useful products (Mevel and Chamroux, 1981; Schroeder, 1978; Wickins, 1985). Austin (1988) concluded that nutrient cycle within the pond is regulated by two main processes, first is assimilation of inorganic nutrient by photosynthetic bacteria including phytoplankton and the second is mineralization by heterotrophic bacteria. The excessive amount of phosphorous compounds do not have adverse effect on the amount of primary production unlike nitrogenous substance *e.g*. ammonia and nitrite. Nutrients accumulation after high

nutrient loading is usually found in concrete tanks or lining ponds where nutrient exchange between water and sediment is very limited (Zur, 1981). In aerobic condition, organic matter decomposed to carbon dioxide, water, nitrogen, phosphate and sulfate. Whereas the anaerobic decomposition ends up with hydrogen sulfide, nitrite, ammonia and nitrogen gas which some of these compounds are harmful to cultured organisms and limit the carrying capacity of the culture systems. The amount of organic matters that controls the concentration of downstream nutrients and the buffering capacity of pond was studied by Hung *et al*. (1990). They suggested that the buffering capacity of the pond can be used as the index for organic matter quantity in the water. In addition, the role of bacteria in aquaculture pond is not only related with nutrient cycle but they also act as the food source for aquatic animal (Schroeder, 1978; Intriago and Jones, 1993). Detritus associated with bacteria serve as food for detritivoros (Tuburan *et al*., 1993).

2.12 Nutrient budget research in shrimp pond

The major sources of nutrient input in shrimp pond consist of feed, water inflow, and fertilizer, stocked shrimp and either organic matter or inorganic fertilizer added to the pond as a part of management practice. In addition to those mentioned, it is also important to include the leaching of nutrients from the sediment during the culture. The nutrient output, however, includes harvested shrimp, discharged and drained water, seepage, nutrient accumulated in the sediment bottom, nitrogen loss through volatilization of ammonia and denitrification.

Understanding in nutrient budget is very important information for aquacultural water treatment and water management in shrimp farming. However, the interaction of physical, chemical and biological processes in the pond is extremely complex and therefore hard to be in control. To keep water quality within the safety level, parameters that known affecting shrimp growth and survival must be monitored and kept at minimum concentration.

Briggs and Funge-Smith (1994) reported that feed is the principal contributor of 91-95% of nitrogen input and 38-60% of phosphorus input in shrimp pond. They suggested that the water born loss was less important than the loss through sediment due to low water exchange rate and rapid accumulation of sediment in the shrimp pond. Phillips *et al*. (1993) also reported the loss of 63-78% N and 76-86% P from feed that did not incorporated in to shrimp biomass. The major sink of the nutrient in the intensive shrimp pond is the sediment, accounted by 30% N and 84% P of the total input (Briggs and Funge-Smith, 1994).

Apart from intensive earthen ponds, Lefebvre *et al.* (2001) reported that the lagoon pond allowed the retain of all particulate feces and food waste produced by fish, despite of remineralization (67% of the sediment material). Ammonia was partially transformed into nitrite and nitrate which induced an accumulation of nitrite in the lagoon pond. Ammonia loss represented 16% of the nitrogen input. Nitrogen excretion plus remineralization minus ammonia loss-the nitrogen dissolved part-represented 61.5%, of

which 43% was ammonia at the outflow. Finally, an average of 40 kg N day^{-1} entered the system as dissolved nitrogen in the seawater.

Martin *et al.* (1998) reported that a quantitative and qualitative studies of solid and dissolved waste output were carried out in relation to stocking density, through the study of nitrogen budget in *Penaeus stylirostris* rearing ponds. Stocking densities ranged from 1 to 30 shrimp m^{-2} resulted in different growth performance and the quantity of waste generation was in proportional with the stocking density. Estimation of nitrogen budget show that up to 38.4% of the nitrogen entering the ponds in the inflow and in the feed pellets, were accumulate in the sediment. The calculated output due to diffusion to the atmosphere and to denitrification account for 9.7 to 32.4% of the total nitrogen.

Table 2.1 Summary of nitrogen budget studies in aquaculture ponds.

Accumulated in aquatic animals (%N)	Loss to pond bottom (%N)	Denitrification and volatilization (%N)	Wasted or discharged water (%N)	Species	References
20-23	27.2-34.0	13-30	27	shrimp (*Penaeus monodon*)	Briggs and Funge-Smith (1994)
31.53	22.57	17.4-57.3	-	fish (channel catfish)	Gross *et al*. (2000)
20-26	-	≥ 50	18	fish	Daniel and Boyd (1989)
26	-	-	25	fish (gilthead seabream)	Krom and Neori (1989)
11	89	-	-	fish (carp)	Zur (1981)
26.8	-	57	-	fish (channel catfish)	Boyd (1985)
9.2	16.7-17.2	9-12	-	Red drum fish (*Sciaenop ocellatus*)	Thoman *et al*. (2001)
4-13.4	23-35	16-64	7-12	fish (catfish-tilapia)	Qifeng (1991)
-	-	-	60	fish (channel catfish)	Turker and Boyd (1985)
-	-	-	80	shrimp	Chien (1992)
12.7-25	15.9-38.4	9.7-32.9	7.8-90.1	shrimp (*Penaeus stylirostris*)	Martin *et al*. (1998)
-	-	-	67-78	shrimp	Phillips *et al*. (1993)
27.1-31.5	0.2	-	45	shrimp (*Penaeus monodon*)	Muthuwan (1991)
14.38-28.81	30.53-42.41	26.11-37.59	94.29	shrimp (*Penaeus monodon*)	Satapornvanit (1993)
14	7.9	6.5	80	shrimp (*Penaeus vannamei*)	Teichert-Coddington *et al* (2000)
19	4	16	61	seabass (*Dicentrarchus labrax*)	Lefebvre *et al*.(2001)
-	60	-	-	fish	Jamu and Piedrahita (2002)
22.8-30.7	14.1-52	5.2-36	14.1-28.4	shrimp (*Penaeus monodon*)	Thakur and Lin (2003)

Table 2.2 Summary of phosphorus budget studies in aquaculture ponds.

Accumulated in aquatic animals (%P)	Loss to pond bottom (%P)	Unaccounted phosphorus (%P)	Wasted or discharged water (%P)	Species	References
13.0	82.1-86.2		92.3-95.1	shrimp (*Penaeus monodon*)	Briggs and Funge-Smith (1994)
-	-		82	fish	Daniel and Boyd (1989)
21	-		75	fish (gilthead seabream)	Krom and Neori (1989)
32	68		-	fish (carp)	Zur (1981)
30	-		-	fish (channel catfish)	Boyd (1985)
6	≥ 84	10	10	shrimp (*Penaeus monodon*)	Funge-Smith and Briggs (1998)
5-44	34-51	3	4-5	fish (catfish-tilapia)	Qifeng (1991)
-	-	5.3-19.7	60	fish (channel catfish)	Turker and Boyd (1985)
8.2-9.7	61.9	-	24.3-27.3	shrimp (*Penaeus monodon*)	Muthuwan (1991)
4.07-8.34	29.1-39.92	35.54-47.7	91.06	shrimp (*Penaeus monodon*)	Satapornvanit (1993)
-	-		76-86	shrimp	Phillips *et al*. (1993)
9	4.5	30.8	55.7	shrimp (*Penaeus vannamei*)	Teichert-Coddington *et al*. (2000)
10.5-12.8	38.8-66.7	5.3-19.7	12.4-28.9	shrimp (*Penaeus monodon*)	Thakur and Lin (2003)

2.13 Use of algae for water treatment in aquaculture

Species of algae suitable for integrated aquaculture may differ depending on the type of culture operation. Most of algal treatment studies were performed using macroalgae (seaweed). Neori *et al.* (1996) found that for treating fish culture effluent with tank cultured seaweeds, the green algae *Ulva* was highly effective, but *Gracilaria* performed poorly. In Chile, *G. chilensis* cultures site located close to salmon cages exhibited an increase in seaweed production (Truell *et al*. 1997).

Various cases of integrating seaweed cultivation with fish culture have been successfully performed. Buschmann *et al.* (1994) illustrated that effluent from intensive tank cultures of salmon in Chile effectively promoted in the production of *G. chilensis* in tank cultures, although they noted that epiphytes were a problem. In Sweden, Haglund and Pedersen (1993) reported that *G. tenuistipitata* worked well in co-cultivation with rainbow trout, particularly during the warmer months of the year. In Israel, the green alga *Ulva lactuca* was found to be an attractive candidate for production with effluent from the culture of the gilthead bream *Sparus aurata* (Vandermuelen and Gordin, 1990). Also, Troell *et al.* (1997) reported that, in the coastal waters of Chile, the *G. chilensis* thalli within 10 m from salmon cages were 40% higher than those 150 m from the cages.

Jones *et al.* (2001) studied the effect of integrated treatment by sedimentation, oyster filtration and macroalgae absorption on effluent from water shrimp culture pond. They reported that the combined effect of the sequential treatments resulted in significant

reduction in the concentration of all parameters measured. High rates of nutrient regeneration were observed in control tanks which did not contain oysters or macroalgae. They concluded that overall, improvements in water quality (final percentage of the initial concentration) were as follows: TSS (12%); total N (28%); total P (14%); NH_4^+ (76%); NO_3^- (30%); PO_4^{3-} (35%); bacteria (30%); and chlorophyll-*a* (0.7%).

2.14 Role of phytoplankton in intensive aquaculture

Photosynthesis by phytoplankton is the primary source of oxygen in most enclosed aquaculture pond (Boyd, 1979). A moderate concentration of phytoplankton is desirable from that stand point of view of water quality. However, as noted by Boyd (1979), die-off phytoplankton related with fish kill is commonly found in fish pond and other small eutrophic bodies of water. Phytoplankton die-offs are most devastating when the phytoplankton crop is so large that decomposition of the dead cells consumes all or most of the oxygen in the water. Plankton are beneficial to fish culture because they utilize nitrogenous wastes that toxic to the fish, consume carbon dioxide and produce oxygen which is necessary for fish respiration. However, because plankton at night also consume most of the oxygen they produce in the daytime, and their growth rate may change dramatically within a of few hours, farmers have to rely on mechanical means of aeration to prevent low dissolved oxygen in the early morning. Researches confirmed that aeration promotes channel catfish production by reducing stress and disease occurrence and improving growth, survival and feed efficiency (Avnimelech and Zohar, 1986; Zang and Boyd, 1988). Neori *et al*. (1996) found that the extreme values of the

water quality parameters, occurred during the bloom and crash cycle of phytoplankton were relate with the greatest damage to fishes.

Referring Redfield equation Pruder (1986) reported that each gram of carbon fixed in photosynthesis, 2.6 g of oxygen is produced and each gram of organic material decomposed, 1.2 g of oxygen consumed. Silver (1992) described that nutrients in marine systems are ammonia and other nitrogenous compounds although in some cases phosphate could be a factor in marine eutrophication. Boyd (1990) mentioned that nitrogen is more important limiting factor in brackish-water ponds than in the freshwater ponds. If there was no limitation of nutrient, light then became the limiting factor to algal growth. In most fishponds, the rate of gross productivity are the highest near water surface and decline rapidly with depth because the dense phytoplankton standing crop reduces the light penetration (Lin, 1986). As the major source of oxygen production is photosynthesis, and oxygen consumption is from algal and bacterial respiration, the balance between heterotrophic and phototrophic organisms is important in aquatic system stability (Pruder, 1986). Algae assimilate ammonia and other potentially toxic nitrogenous metabolic waste product and help to maintain dissolved oxygen concentration (Law and Malecha, 1981; Boyd, 1982). In extensive shrimp culture pond, the photosynthesis and decomposition process by photoautotrophs is disassimilated mainly by aerobic pathway and pond condition are relatively stable (Pruder, 1986).

CHAPTER III

EFFECT OF SALINITY ON AMMONIUM AND NITRATE UPTAKE KINETICS OF *Spirulina platensis*

3.1 INTRODUCTION

Microalgae has been intensively studied as an alternatively biological nutrients removal for wastewater treatment system, especially in the tropical and subtropical regions. The advantage of algal system is that algae use solar energy to produce oxygen and take up inorganic nutrients such as nitrogen and phosphorus from wastewater and assimilate them their growth. Although there were several publications on using algae for nutrients removal from wastewater (de la Noue *et al.*, 1992; Lau *et al.*, 1995; Lau *et al.*, 1996; Lefebvre *et al.*, 1996; Craggs *et al.*, 1997; Ahn *et al.*, 1998; Chopin *et al.*, 2001), research on algal physiology especially on nutrient uptake rate has been rarely conducted.

Nitrogen assimilation in algae is a result from several uptake mechanisms. In detail, ions transport through algal cell membrane is related to four main pathways that are: (1) adsorption of ions to certain component of the cell wall (2) passive transport by diffusion in which charged ions movement across the membrane arises from electrical-potential differences and/or chemical-potential differences and the combined electrochemical potential gradient (3) facilitated diffusion which is the passive diffusion by electrochemical gradient but the rate of transport is limited by carrier proteins at the

outer membrane and (4) active transport that need energy to drive ions against an electrochemical-potential gradient. Nutrient uptake kinetics is depend on which uptake mechanism is being used. For passive diffusion, the transport rate is a directly proportion to the external concentration. Facilitated diffusion and active transport, on the other hand, exhibit a saturation of the transport or uptake rate when the membrane carriers are saturated. At this point, uptake rate can not be further increase even when the external concentration of ions increase.

In this study, nitrogen uptake rate was evaluated by measuring the disappearance of nitrogen compounds (ammonium or nitrate) from the medium with conventional chemical analysis rather than radioactive or stable-isotope uptake. There are two basic approaches to following the disappearance of the nutrient from the medium as showed in **Fig. 3.1**, the first is to spike the culture with the nutrient of interest and follow the nutrient disappearance for several hours until nutrient exhaustion occurs and the second approach is a short incubation in many containers with different concentration of nutrients. The second method was then chosen in the present study because it is generally used to estimate the maximal uptake rate.

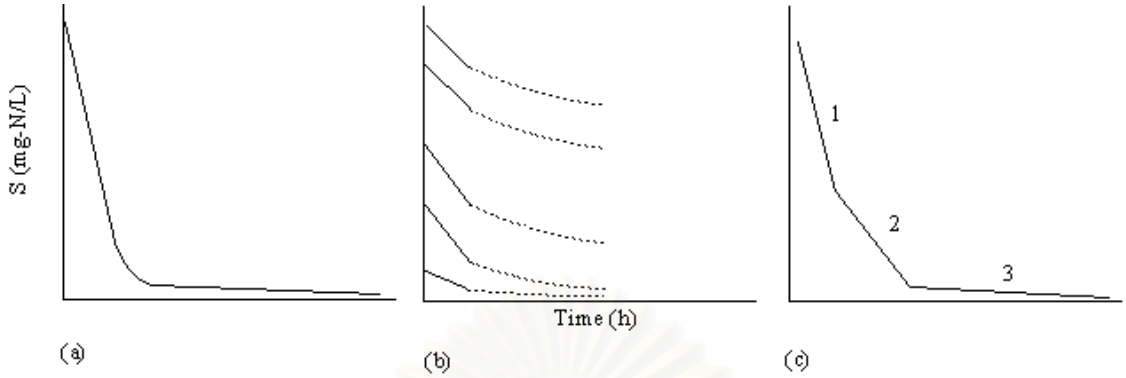

Figure 3.1 Hypothetical time series showing nutrient disappearance from the medium using the following methods: (a) the perturbation method, where saturated uptake is linear with time; (b) the multiple-container, constant-incubation time method; (c) the perturbation method, where saturated uptake is nonlinear with time. Phase 1 is the enhanced or rapid uptake, possibly due to filling of intracellular pool (s); phase 2 may represent an assimilation rate (also referred to as V_i); phase 3 is the final depletion of the limiting nutrient from medium. (Lobban and Harrison, 1994).

Algal nutrient uptake rate is not only affected by the availability of nutrients but it also depended on physical factors such as salinity, light, temperature and others (Lobban and Harrison, 1994). In practical, salinity is the only parameter in the aquaculture system that could be successfully controlled while other parameters such as light and temperature were depended on natural conditions. Effect of salinity stress on *Spirulina* as studied by Zeng and Vonshak (1998) confirmed that the adaptation process to salinity-stress in *Spirulina* was an energy consuming process. Salinity could enhance

photoinhibition of photosynthesis through direct effects on Photosystem II reaction center. Since the main objective of this thesis was to apply *Spirulina* for nitrogen removal in seawater environment, the effect of salinity on nitrogen uptake kinetics of *Spirulina* was therefore required. The aim of this chapter was to evaluate the effect of different salinities (0, 15 and 30 psu) on ammonium and nitrate uptake kinetics of *Spirulina* using Michaelis-Menten equation.

3.2 MATERIALS AND METHODS

3.2.1 S. platensis culture

Stock culture of *S. platensis* was obtained from the Institute of Food Research and Product Development, Kasetsart University, Thailand. *S. platensis* was cultured in 30 psu (~0.51 M NaCl) Zarrouk medium (Zarrouk, 1966) at 25-28°C under continuous illumination at 100 μmol photon m^{-2} s^{-1} with fluorescent bulbs.

3.2.2 Experimental procedure

Concentrated *Spirulina* cells were incubated in nitrogen free Zarrouk's medium for 24 hours before starting the nutrient uptake experiments. The experiments were carried out in 250 ml flasks containing 150 ml Zarrouk's medium (Appendix A) with various nitrogen concentrations.

The objective of this experiment was to study the effect of salinity on ammonium and nitrate uptake kinetics of *Spirulina* in laboratory condition. Three levels of salinity (0, 15 and 30 psu) were used in conjunction with various nitrogen concentrations of both ammonium and nitrate trials. Nitrogen uptake kinetics were determined by measuring the rate of disappearance of ammonia or nitrate from the water. For ammonium uptake trial, six concentrations of NH_4Cl (2, 4, 6, 8, 10 and 12 mg NH_4-N

L^{-1}) were used. Furthermore, five concentrations of KNO_3 (5, 10, 20, 50 and 100 mg NO_3-N L^{-1}) were used in nitrate uptake trial. Those nitrogen concentrations were resemble the concentration that probably found in shrimp culture tanks. All treatments of all trials were performed in triplicate flasks containing modified Zarrouk's medium with desired nitrogen sources.

After incubated in various nitrogen concentrations as previous mentioned, water samples were taken from the culture flasks after 0, 0.17 (15 minutes), 0.5, 1, 2, 4, 8, 12, 16, 20 and 24 hours for ammonium or nitrate analysis. Water samples were filtered through 1.2 microns Whatman glass fiber filter (GF/C) prior to water analysis. Determination of ammonium and nitrate was according to Parson *et al*. (1989). Chlorophyll-*a* was also examined using a method modified from Bennet and Bogorad (1972) and Parson *et al*. (1989). Detail of the chemical analysis is shown in Appendix B. Linear reduction of nitrogen disappearance during the first hour, confirmed by regression analysis, was used for the uptake rate estimation. Finally, calculation of K_m and V_{max} in the Michaelis-Menten equation was performed with Hanes-Woolf's transformation (Kopozak, 1994; Qi and Zhu, 1994; Taylor and Rees, 1999).

3.3 RESULTS AND DISCUSSION

3.3.1 Ammonium uptake of S. platensis

Figure 3.2-3.4 shows the disappearance of ammonium from the culture medium which was a result of ammonium uptake by nitrogen-starved *S. platensis*. Note that the unit of ammonium uptake in Fig. 3.2-3.4 in mg NH_4-N/mg Chl-*a* because of different initial chlorophyll concentrations. It was found that ammonium concentration was rapidly decreased in the first hour, thereafter, ammonium uptake rate was similarly declined in all treatments. Regression analysis in **Fig. 3.5-3.7** showed that ammonium reduction during the first hour was in linear range with R^2 more than 0.9 in most treatments. Hence, uptake rate was then calculated using the data from the first hour only (Appendix C).

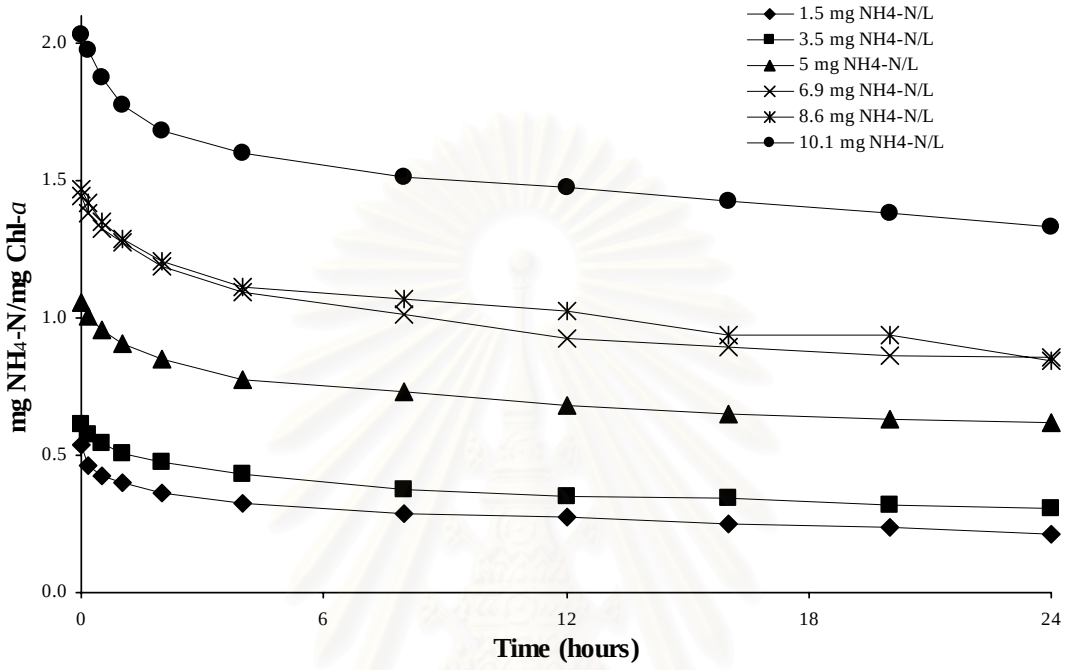

Figure 3.2 Ammonium uptake by *S. platensis* cultured in modified Zarrouk's medium supplemented with various ammonium concentrations at 0 psu salinity.

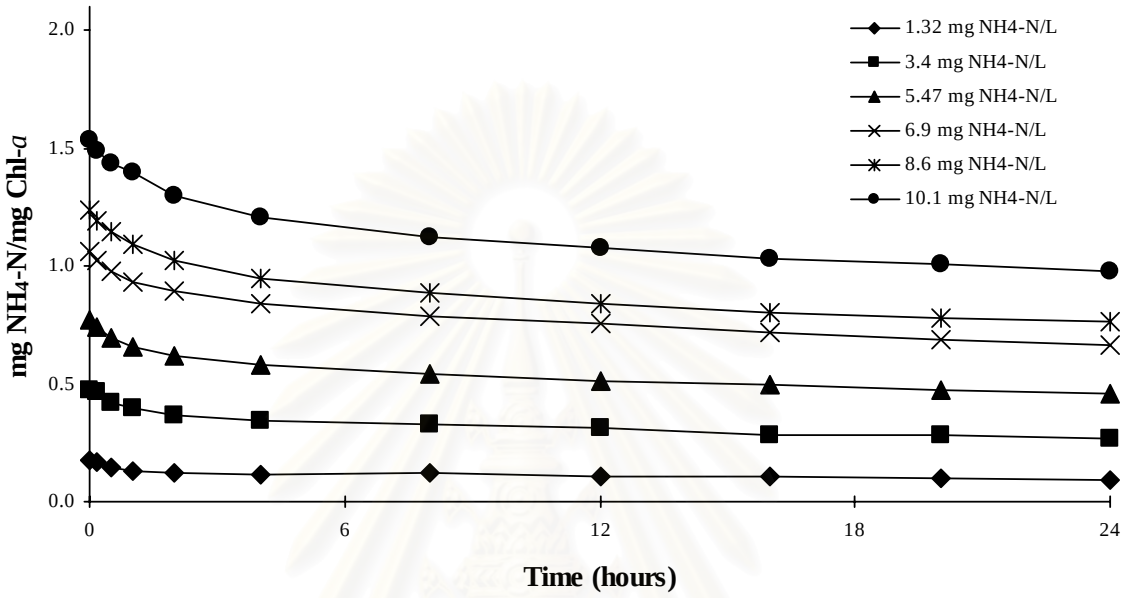

Figure 3.3 Ammonium uptake by *S. platensis* cultured in modified Zarrouk's medium supplemented with various ammonium concentrations at 15 psu salinity.

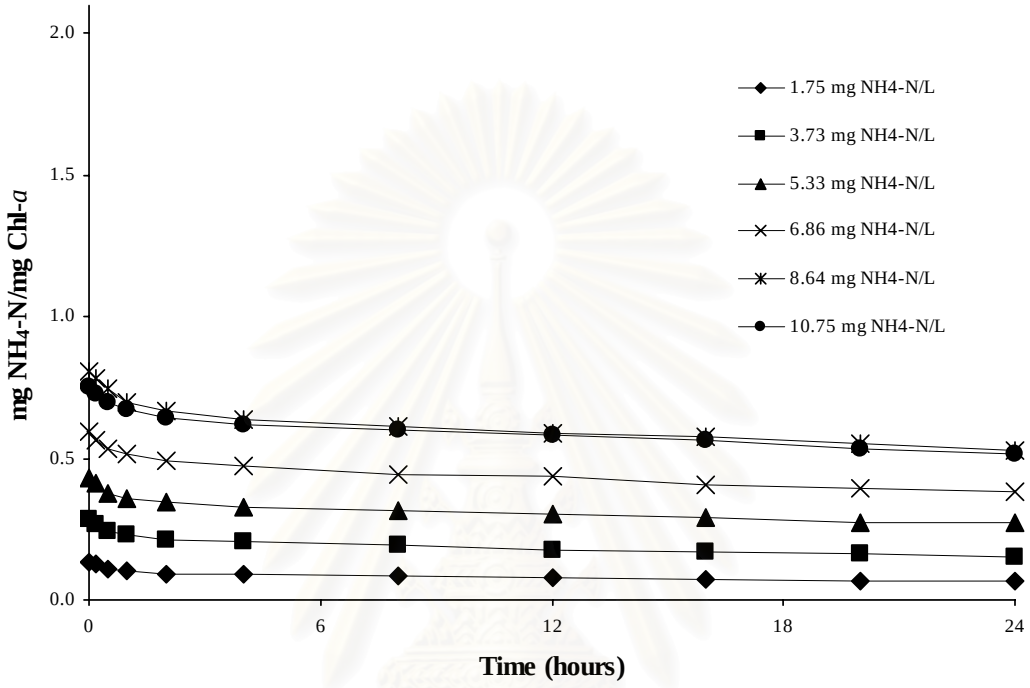

Figure 3.4 Ammonium uptake by *S. platensis* cultured in Modified Zarrouk's medium supplemented with various ammonium concentrations at 30 psu salinity.

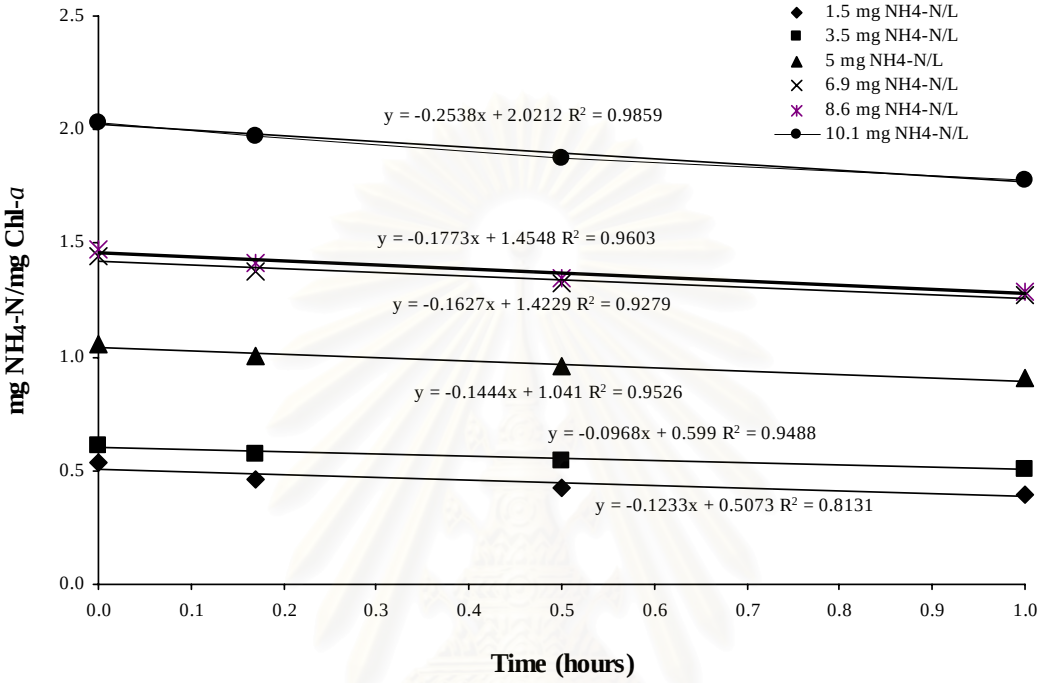

Figure 3.5 Linear reduction of ammonium from 0 psu culture medium during the first hour according to regression analysis.

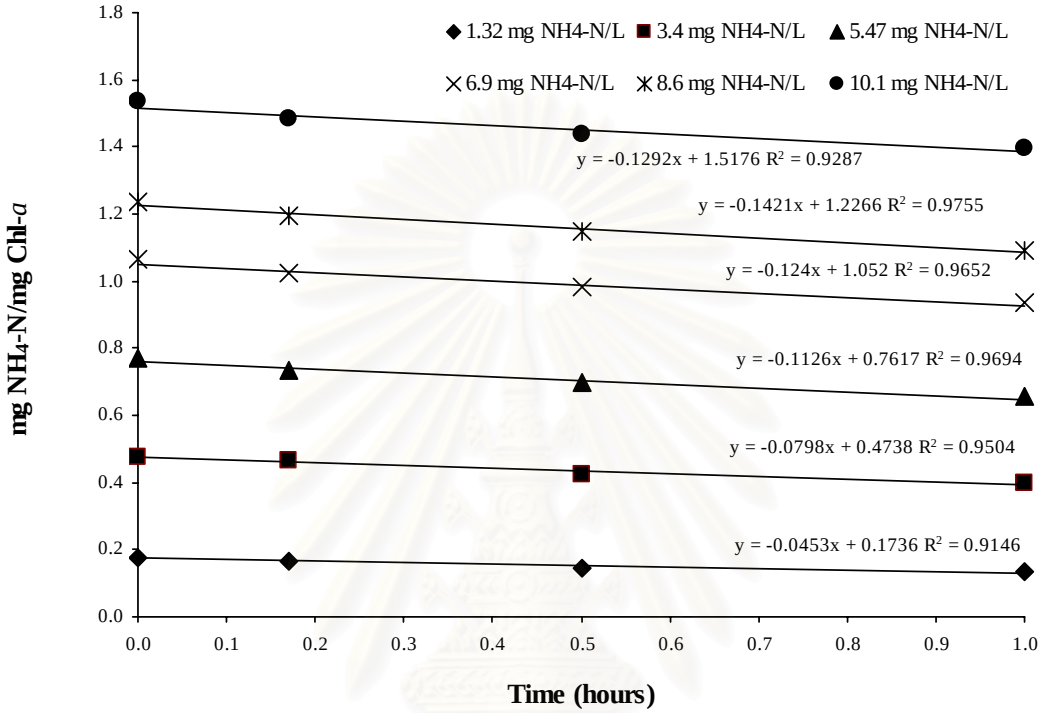

Figure 3.6 Linear reduction of ammonium from 15 psu culture medium during the first hour according to regression analysis.

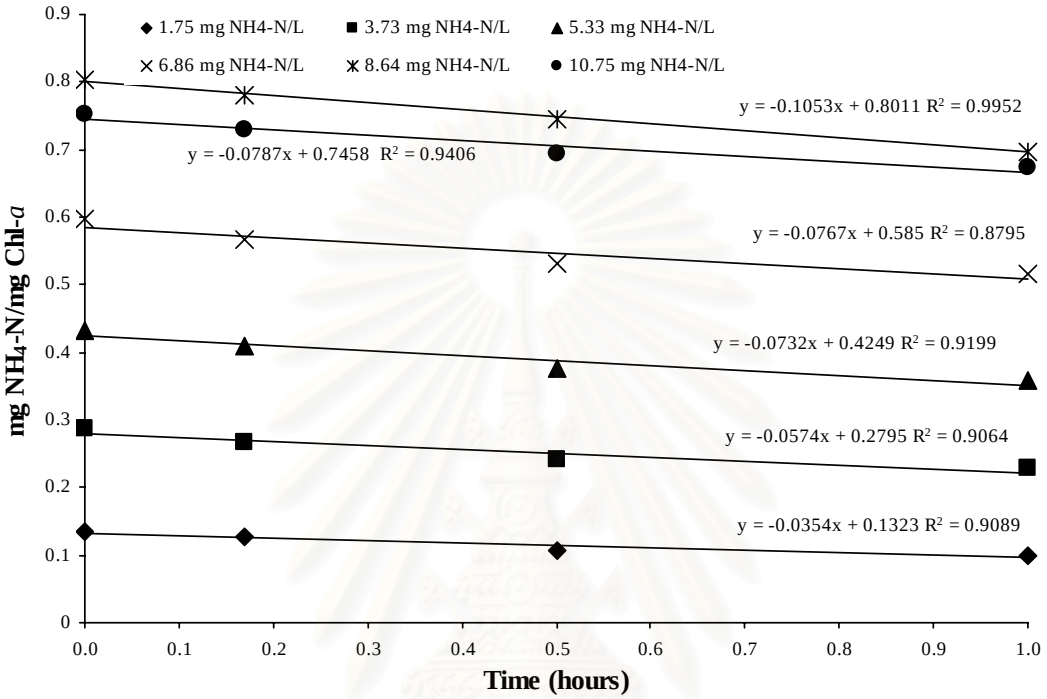

Figure 3.7 Linear reduction of ammonium from 30 psu culture medium during the first hour according to regression analysis.

The results from this trial suggested that salinity had strong affect to ammonium uptake rate. Comparison of the uptake rate in **Table 3.1** shows that ammonium uptake of *S. platensis* in 30 psu medium was less than half of that found in 0 psu while the uptake rate of 15 psu medium was in the middle.

Table 3.1 Ammonium uptake rate of *S. platensis* at different salinity.

Initial ammonium concentration* (mg NH_4-N L^{-1})	**Ammonium uptake rate (mg NH_4-N mgChl-*a*$^{-1}$ h^{-1}) of different salinity culture**		
	0 psu	**15 psu**	**30 psu**
1.5	0.123	0.045	0.035
3.5	0.097	0.079	0.057
5	0.144	0.112	0.073
7	0.163	0.124	0.077
8	0.177	0.142	0.079
10	0.254	0.129	0.105

*Initial ammonium concentrations showed in this table were the approximated values since the certain values from ammonium analysis were slightly different in each treatment.

Evaluation of ammonium uptake kinetics was performed by Hanes-Woolf plot between S/V and S. Since the rate of ammonium uptake (V) was a saturable function of ammonium concentration (S), Hanes-Woolf plot in **Fig. 3.8** allowed the calculation of V_{max} and K_m using simple linear regression equation. The results of kinetic constants derived from Hanes-Woolf plot are showed in **Table 3.2**. Thereafter,

calculated K_m and V_{max} values were used to estimate Michaelis-Menten plot of ammonium uptake kinetics as showed in **Fig. 3.9**.

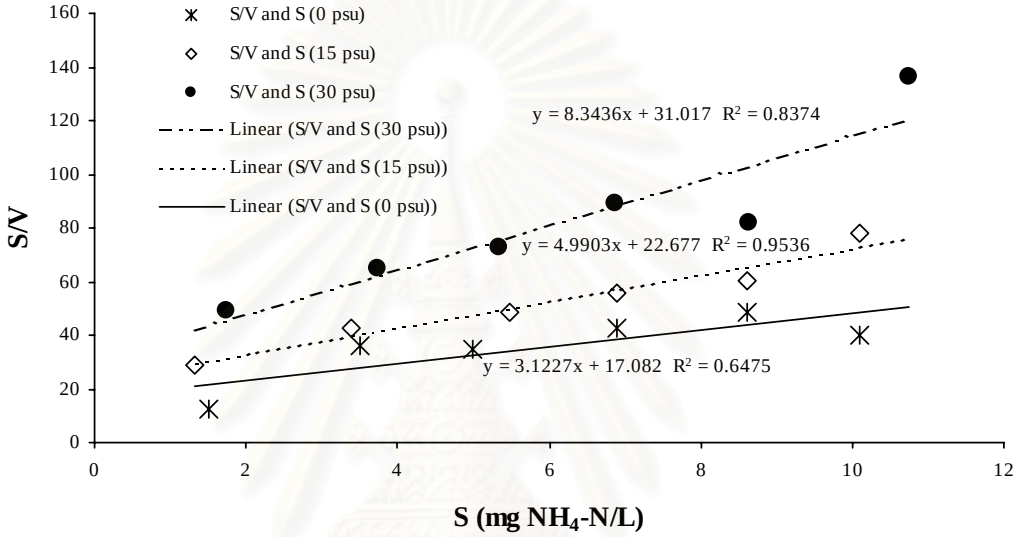

Figure 3.8 Hanes-Woolf plot (plotted between S/V and S) of various ammonium concentrations at 0, 15, and 30 psu salinity of *Spirulina platensis*: [Σ] 0 psu salinity, [ ] 15 psu salinity, and [●] 30 psu salinity.

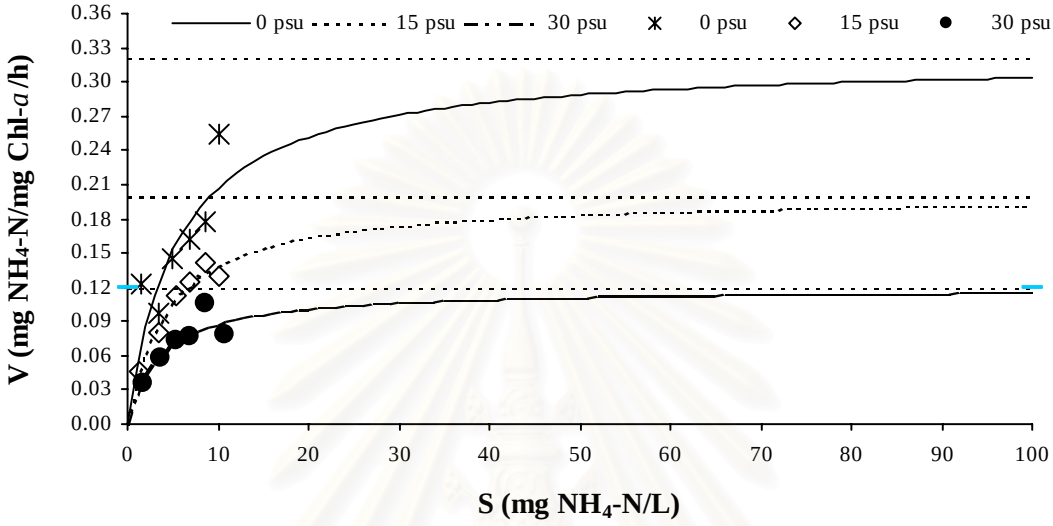

Figure 3.9 Ammonium uptake kinetics of *S. platensis* in 0, 15 and 30 psu salinity of Zarrouk's medium, as illustrated by Michaelis-Menten equation.

Table 3.2 . Kinetic constants of ammonium uptake by *S. platensis* at various salinities.

Kinetic constants	**Salinity**		
	0 psu	**15 psu**	**30 psu**
K_m (half-saturation constant; mg NH_4-N L^{-1})	5.470	4.544	3.717
V_{max} (calculated maximum uptake rate; mg NH_4-N mgChl-*a*$^{-1}$ h^{-1})	0.320	0.200	0.120
Maximum uptake rate found in this experiment (mg NH_4-N mgChl-*a*$^{-1}$ h^{-1})	0.254	0.142	0.105

3.3.2 Nitrate uptake of S. platensis

Nitrate disappearance from modified Zarrouk's culture medium by nitrogen-starved *S. platensis* resembled the pattern of ammonium uptake in which nitrate concentration was rapidly decreased during the first hour **(Fig. 3.10-3.12)**. Note that the unit of nitrate uptake in Fig. 3.10-3.12 in mg NO_3-N/mg Chl-*a* because of different initial chlorophyll concentrations. As shown in **Fig. 3.13-3.15**, calculation of nitrate uptake rate using regression analysis showed a good linear relationship (with R^2 more than 0.9) between amount of nitrate versus time.

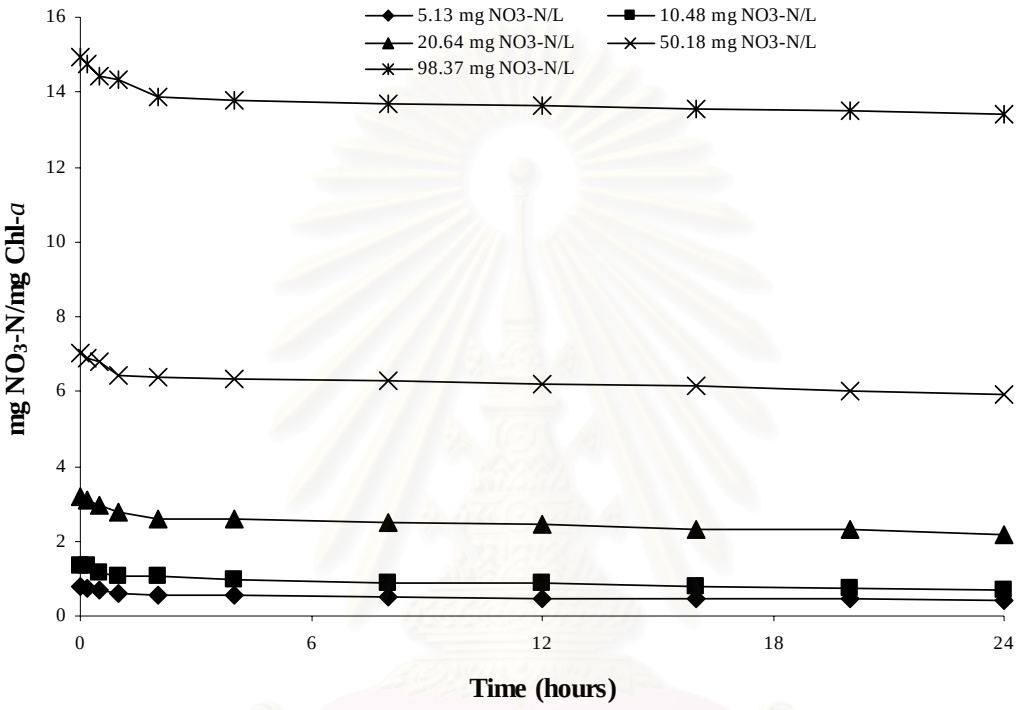

Figure 3.10 Nitrate uptake by *S. platensis* cultured in modified Zarrouk's medium supplemented with various nitrate concentrations at 0 psu salinity.

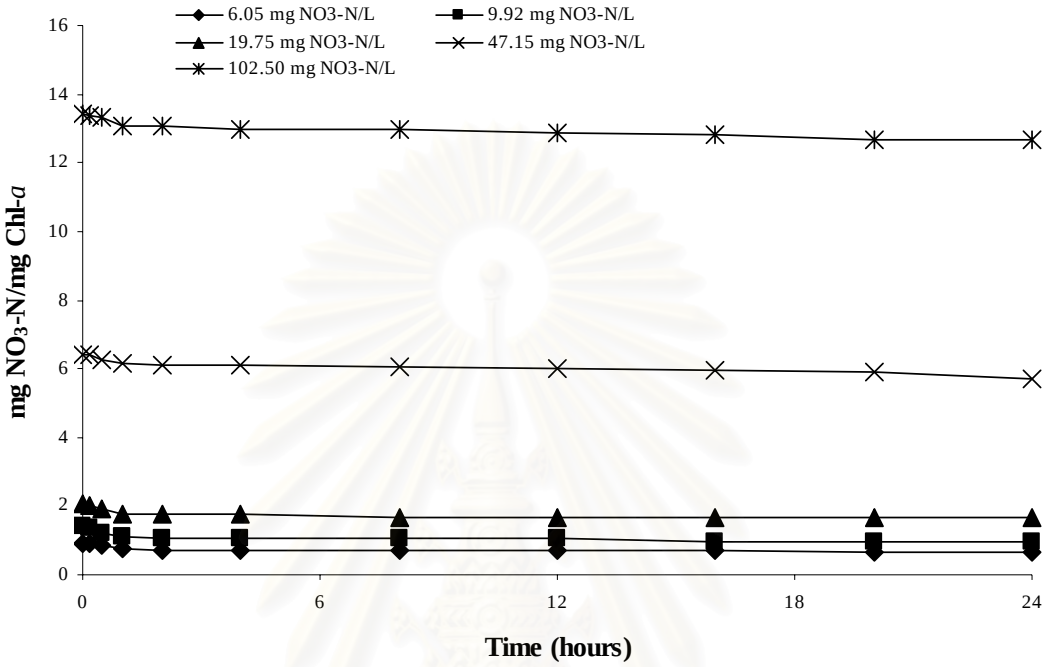

Figure 3.11 Nitrate uptake by *S. platensis* cultured in modified Zarrouk's medium supplemented with various nitrate concentrations at 15 psu salinity.

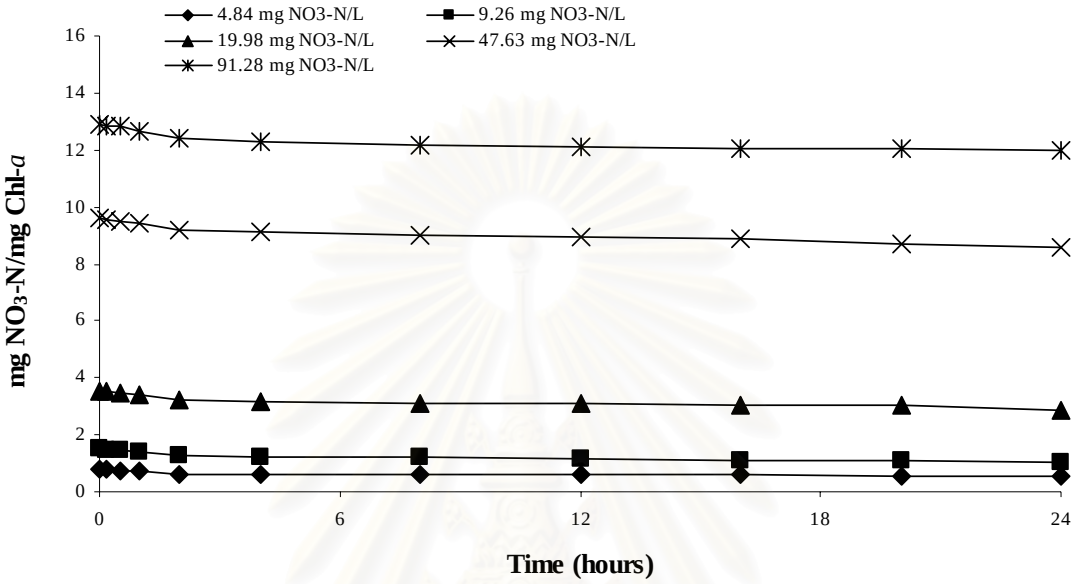

Figure 3.12 Nitrate uptake by *S. platensis* cultured in modified Zarrouk's medium supplemented with various nitrate concentrations at 30 psu salinity.

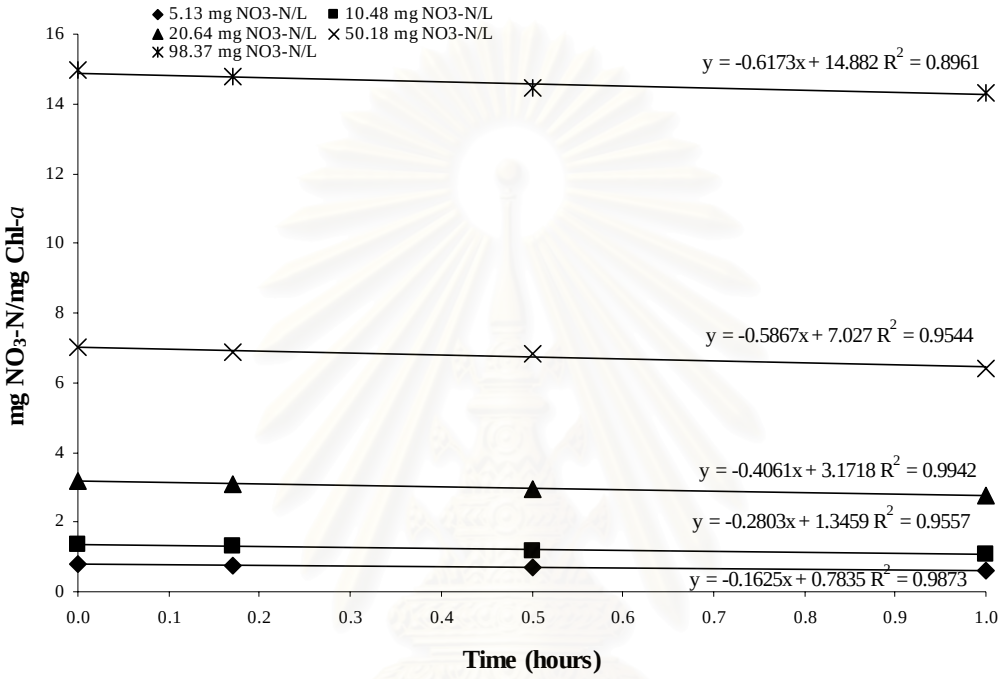

Figure 3.13 Linear reduction of nitrate from 0 psu culture medium during the first hour according to regression analysis.

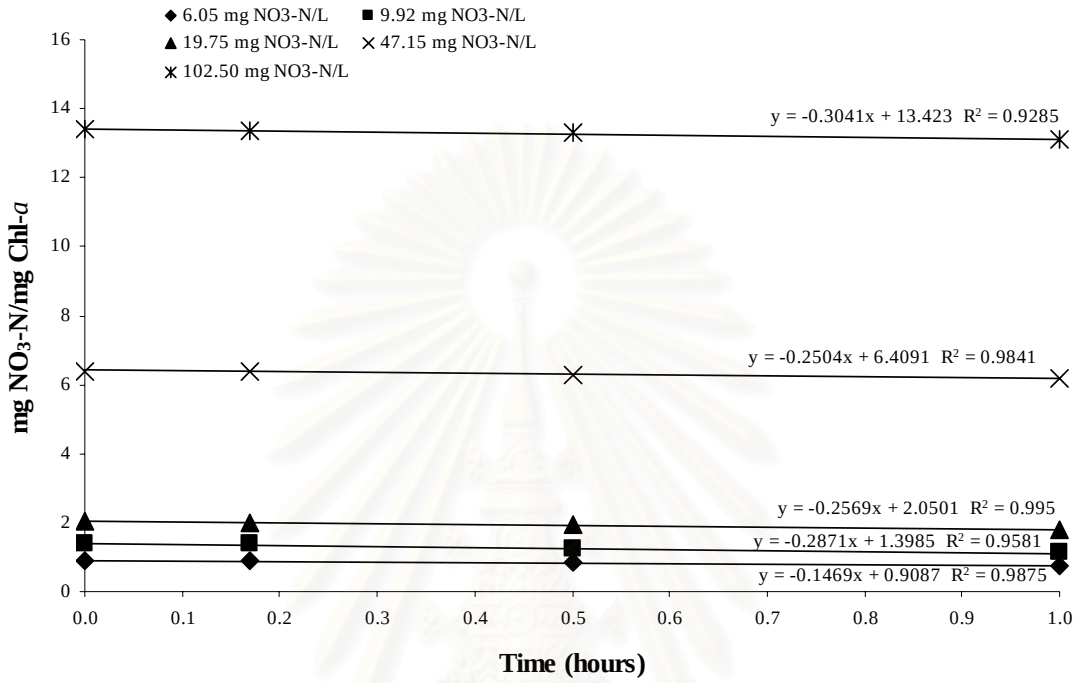

Figure 3.14 Linear reduction of nitrate from 15 psu culture medium during the first hour according to regression analysis.

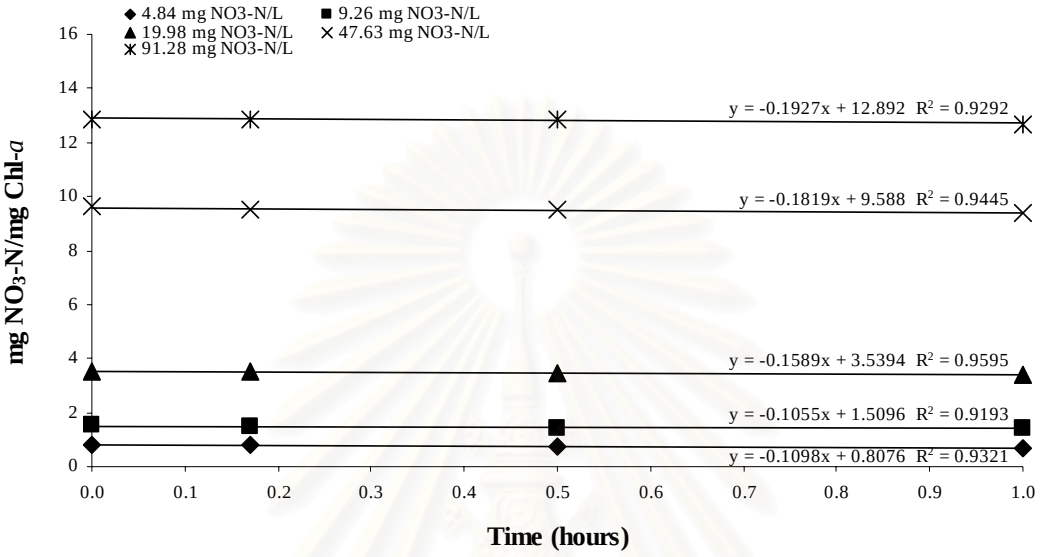

Figure 3.15 Linear reduction of nitrate from 30 psu culture medium during the first hour according to regression analysis.

It was found that salinity also had strong affect to nitrate uptake rate of *S. platensis*. **Table 3.3** shows that the nitrate uptake rates of *S. platensis* cultured in 0 psu salinity medium were almost three folds higher than in 30 psu medium. However, the nitrate uptake rate of *S. platensis* in 15 psu culture medium was closer to the rate of 30 psu culture than the 0 psu culture.

Table 3.3 Nitrate uptake rate of *S. platensis* at different salinities.

Initial nitrate concentration* (mg NO_3-N L^{-1})	**Nitrate uptake rate (mg NO_3-N mgChl-*a*$^{-1}$ h^{-1})**		
	0 psu	**15 psu**	**30 psu**
5	0.162	0.147	0.109
10	0.280	0.287	0.105
20	0.406	0.257	0.159
50	0.587	0.250	0.182
100	0.617	0.304	0.193

*Initial nitrate concentrations showed in this table were approximated values since the certain values from nitrate analysis were slightly different in each treatment.

Evaluation of nitrate uptake kinetics was performed by the same manner as ammonium uptake kinetics using Hanes-Woolf plot **(Fig. 3.16)**.

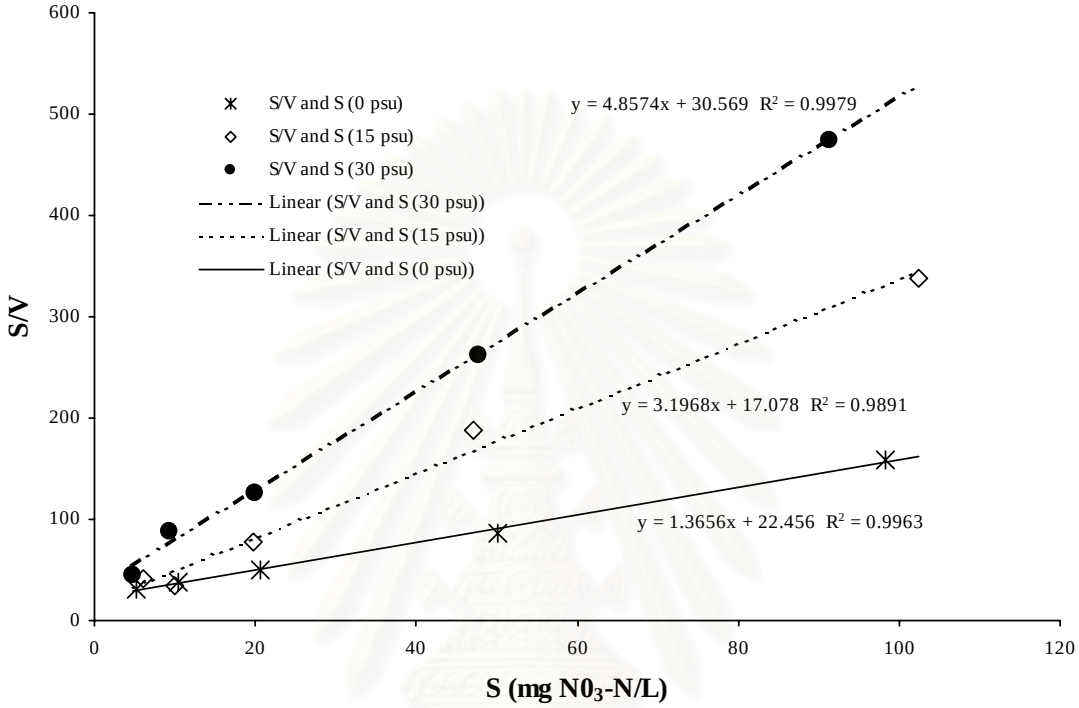

Figure 3.16 Hanes-Woolf plot (plotted between S/V and S) of variuos nitrate concentrations at 0, 15, and 30 psu salinity of *S. platensis*: [Σ] 0 psu salinity, [ ] 15 psu salinity, and [●] 30 psu salinity.

Thereafter, the results of kinetic constants (K_m and V_{max}) derived from Hanes-Woolf transformations are showed in **Table 3.4** following by the Michaelis-Menten kinetics plot of nitrate uptake in **Fig. 3.17**.

Table 3.4 Kinetic constants of nitrate uptake by *S. platensis* at various salinities.

Kinetic constants	**Salinity**		
	0 psu	**15 psu**	**30 psu**
K_m (half-saturation constant; mg NO_3-N L^{-1})	16.444	5.342	6.293
V_{max} (calculated maximum uptake rate; mg NO_3-N mgChl-*a*$^{-1}$ h^{-1})	0.732	0.313	0.206
Maximum uptake rate found in this experiment (mg NO_3-N mgChl-*a*$^{-1}$ h^{-1})	0.617	0.304	0.193

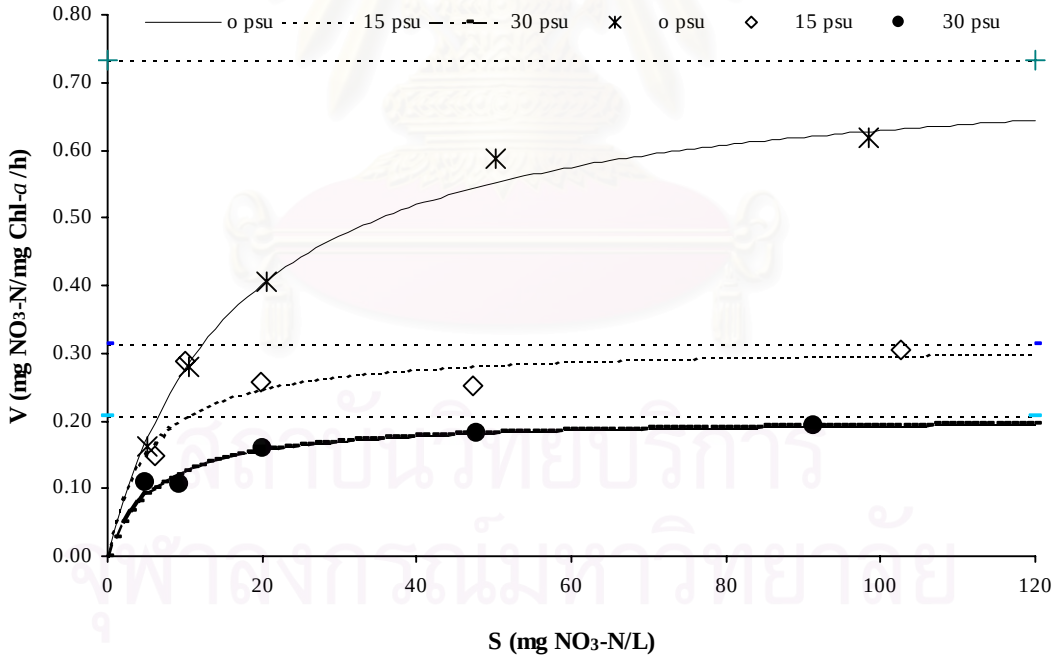

Figure 3.17 Nitrate uptake kinetics of *S. platensis* in 0, 15 and 30 psu salinity of Zarrouk's medium, as illustrated by Michaelis-Menten equation.

3.3.3 Discussion

In most aquaculture tanks or lined ponds that do not contain bottom sediment, nitrate accumulation is usually found as a result of nitrification process. Therefore, nitrate concentration more than 100 mg NO_3-N L^{-1} was possibly found (Menasveta *et al*., 2001). On the other hand, ammonium concentration is usually not higher than 5-10 mg NH_4-N L^{-1} since ammonium and ammonia are usually converted into nitrite and nitrate in most well aerated tanks. Although there were a lot of reports on using *Spirulina* for nutrient removal from wastewater (Chung *et al*., 1978; Oron *et al*., 1979; Seshadri and Thomas, 1979; Tanticharoen *et al*., 1990; Fedler *et al*., 1993; Phang *et al*., 2000), the kinetics study of ammonium and nitrate uptake has never been reported. Evaluation of ammonia and nitrate uptake kinetics of *S. platensis* in this study was performed within the range of nitrogen concentrations that could be found in the normal shrimp culture practice. These ranges, including 1.5-10 mg NH_4-N L^{-1} and 5-100 mg NO_3-N L^{-1}, were much higher than most nitrogen uptake studies published elsewhere.

The results from this experiment indicated that nitrogen-starved *S. platensis* could uptake nitrogen in both ammonium and nitrate forms. Rapidly disappearance of nitrogen from the medium was found only in the first hour, thereafter the rate was soon decrease. This reduction pattern was resembled with that mentioned in Ryther *et al*. (1981) and Jones *et al*. (2001). It has to be noted that *S. platensis* used in this experiment was already adapted to the culture medium (Zarrouk's medium) containing nitrate as a sole nitrogen source. Therefore, lower ammonium uptake rate might be more or less

affected by intracellular nitrate storage. To reduce this effect, the algal cells were incubated in nitrogen-free Zarrouk medium for 24 hours before starting the experiment.

It is generally known that ammonium is a preferred nitrogen source for pico- and nanoplankton and less by larger phytoplankton. Larger phytoplankton would be more dependent on nitrate since it use intracellularly stored nitrate as a reservoir for future assimilation and growth (Dortch, 1982; Stolte and Riegman, 1996). In some algae with simultaneous ammonium and nitrate supplement, ammonium was taken up faster than nitrate because of feedback inhibition of nitrate reductase enzyme by high concentration of intracellular ammonium. Although ammonium uptake is likely a passive uptake process, slightly leakage of ammonium via the permeability of the plasma membrane was also mentioned in some literature such as Stolte and Riegman (1996) but only at very small amount (less than 1% of intracellular ammonia).

It is known that *Spirulina* could grow in seawater (Materassi *et al*., 1984; Tredici *et al*., 1986). In this study, high salinity (15 and 30‰) had strong affect to nitrogen uptake rate in both ammonium and nitrate forms but salinity seemed to have more affect to nitrate uptake than ammonium uptake. However, since the range of ammonium and nitrate concentrations in both trials were different, the most acceptable comparison must came from the same nitrogen concentrations of 5 and 10 mg-N L^{-1} only. As showed in Tables 3.1 and 3.3, ammonium uptake rate of 0.254, 0.129 and 0.105 mg NH_4-N mgChl-a^{-1} h^{-1} for *S. platensis* cultured in 0, 15 and 30 psu medium, respectively,

were slightly lower than nitrate uptake rate of 0.280, 0.287 and 0.105 mg-NO_3-N mgChl-a^{-1} h^{-1} for *S. platensis* cultured with the same salinity.

Zeng and Vonshak (1998) suggested that *Spirulina* has lower photosynthetic activity when growing in high salinity, especially after a sudden increased of salinity. Under high salinity condition, *Spirulina* growth rate and photosynthesis were decreased while respiration rate of the alga was found increased. Decrease in the growth rate by salt stress was also studied in other cyanobacteria such as *Anacystis* (Vonshak and Richmond, 1981) and *Nostoc* (Blumwald and Tel-Or, 1982). Molitor *et al.* (1986) found that the increase in respiration rate of *Anacystis nidulans* grown in NaCl-enriched medium was due to the enhanced activity of the plasma membrane cytochrome oxidase. It was also suggested by Gabbay-Azaria *et al.* (1992) that Na^+ and Cl^- triggered or activated electron transport of the respiratory system in the cells. Increase in respiration rate after salinity stress was also reported in various cyanobacteria (Fry *et al.* 1986; Gabbay-Azaria *et al.* 1992; and Komenda and Masojidek, 1995). This was then concluded that salinity adaptation was an energy consuming process. Generally, effect of salinity-stress consisted of a relatively short shock stage, following by an adaptation process (Vonshak *et al.*, 1988). In this experiment, *S. platensis* was cultured in the medium with various salinities for many generations before starting nitrogen uptake experiment. Therefore, nitrogen uptake rate in this study was a result from well-adapted cells.

In general, nitrogen uptake rate of most algae was a function of nitrogen concentration and could be evaluated using Michaelis-Menten kinetics equation. This was confirmed by many publications of nitrate uptake in microalgae such as *Prorocentrum mican*, *Chaetoceros lorenzianus*, *Biddulphia aurita* (Underhill, 1977) and *Asterionela japonica* (Eppley and Thomas, 1969) as well as in macroalgae such as *Laminaria groenlandica* (Harrison *et al*., 1986), *Chaetomorpha linum* (Lavery and McComb, 1991), *Gracilaria pacifera* (Thomas *et al*., 1987) and *Chondrus cripus* (Amat and Braund, 1990). Linear transformation using Hanes-Woolf-plot provided reliable results with strong relationship between S/V and S after regression analysis. This could be used with more confidence than the transformation by plotting 1/V versus 1/S that is commonly used in enzyme kinetic study. The advantage of Hanes-Woolf-plot is that the data (*e.g*. nitrogen concentrations) of equal sequence for example 1, 2, 3,… or 5, 10, 15,… will be transformed into uniformly distributed data between S/V and S axis. On the other hand, data transformation by plotting 1/V and 1/S of those mentioned data will resulted as a group of transformed data mostly located close to 1/V axis. This could possibly cause an error to slope determination after regression analysis **(Fig. 3.18)**. Therefore, most of the nutrient uptake kinetics studies were thus use Hanes-Woolf tranformation to estimate V_{max} and K_m (Williams, 1984; Taylor and Rees, 1999; Topinka, 1978; Qi and Zhu, 1994; Underhill, 1977; Eppley and Thomas, 1969).

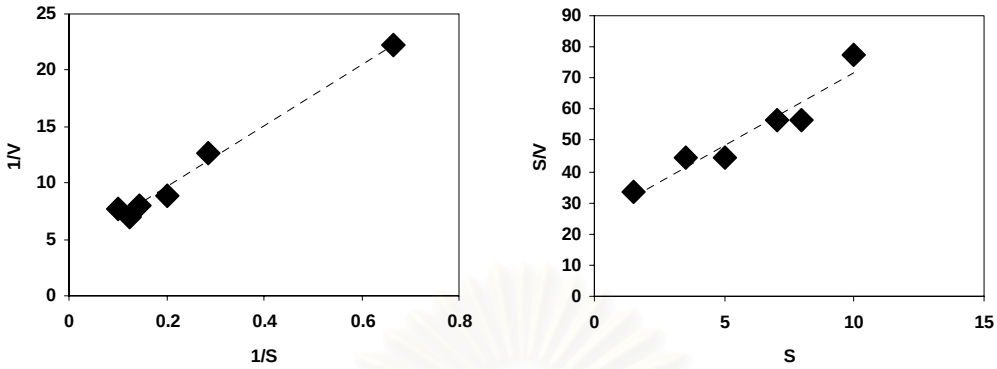

Figure 3.18 Example of linear transformation using 1/V versus 1/S (left) that was commonly used with enzyme kinetics, or S/V versus S (Hanes-Woolf-plot: right) that was used in this study. The original data of both plots came from the same data of ammonium uptake rate at 15 psu salinity (Table 3.1).

Generally, the nutrient uptake rates of phytoplankton were different from species to species (Pasciak and Gavis, 1974) and also depend on environmental condition. Comparison of kinetic constants found in this study with the finding of other publications is shown in **Table 3.5 and 3.6**. As seen in both tables, direct comparison of K_m and V_{max} among several publications was not possible because of the different in measuring unit. In this study, nitrogen uptake rate had to be standardized by chlorophyll unit or dry weight, not by cell number, because *Spirulina* is a multicellular filamentous alga.

Table 3.5 Comparison of kinetic constants of ammonium uptake by algae.

Species	K_m	V or V_{max}	References
Anacystis nidulans and Nostoc muscorum	1-66 (µM)	-	Boussiba *et al*. (1984); Kashyap and Johar (1984)
Phaeodactylum tricornutum	35 (µM)	-	Wright and Syrett (1983)
Cyclotella cryptica	6-39 (µM)	-	Wheeler (1980)
Microalgae and cyanobacteria	1 (µM)	-	Eppley *et al*. (1969); Eppley and Renger., (1974) cited in Kerby *et al*. (1989); Healey., 1977 cited in Kerby *et al*. (1989)
Prorocentrum minimum	8.5 (µM)	V_{max} 0.45 (10^6 µmol. $Cell^{-4}.h^{-1}$)	Pasciak and Gavis (1974)
Cyclotella nama	0.4-1.9 (µM)	V_{max} 4.0-9.0 (10^9 µmol. $Cell^{-4}.h^{-1}$)	Carpenter and Guillard (1971)
Codium fragile	1.5 ± 0.2 (µM)	13.0 (µmol $g_{dw}^{-1}h^{-1}$)	Hanisak and Harlin (1978)
Chondrus crispus	35.5 (µM)	62 (µmol $g_{dw}^{-1}h^{-1}$)	Amat and Braud (1990)
Laminaria. saccharina	-	6.0-8.9 (µmol $g_{dw}^{-1}h^{-1}$)	Ahn *et al*. (1998)
Nereocystis. luetkeana	-	6.6-9.3 (µmol $g_{dw}^{-1}h^{-1}$)	Ahn *et al*. (1998)
Spirulina platensis	**264.44-385.56 (µM) (3.7-5.4 mg NH_4-N L^{-1})**	**V_{max} 0.12-0.32 (mg NH_4-N. mgChl-a^{-1} h^{-1})**	**This study.**

Table 3.6 Comparison of kinetic constants of nitrate uptake by algae.

Species	K_m	V or V_{max}	References
Prorocentrum micans	1.55 (µM)	4.72 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Qi and Zhu (1994)
Chaetoceros lorenzianus	4.50 (µM)	0.48 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Qi and Zhu (1994)
Prorocentrum minimum	8.5 (µM)	0.45 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Pasciak and Gavis (1974)
Cyclotella nana	0.4-1.9 (µM)	4.0-9.0 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Carpenter and Guillard (1971)
Diatom	-	20 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Lehman *et al*. (1975)
Dinoflagellates	-	100 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Lehman *et al*. (1975)
Dunaliella salina	1290.32 (µM) (0.08 g L^{-1})	15.76 ($x10^{-6}$ µmol $cell^{-1}$ h^{-1})	Buranapratheprat *et al*. (1994)
Cyanidium caldarium	<1.0µM - 0.45 mM	-	Fuggi *et al*., 1984 cited in Kerby *et al* (1989)
Laminaria longicruris	4.1 (µM)	9.6 (µmol $g_{dw}^{-1}h^{-1}$)	Harlin and Craigie (1978)
Macrocystis pyrifera	13.1±1.6 (µM)	30.5 (µmol $g_{dw}^{-1}h^{-1}$)	Haines and Wheeler (1978)
Codium fragile	1.9±0.5 (µM)	2.8 (µmol $g_{dw}^{-1}h^{-1}$)	Hanisak and Harlin (1978)
Enteromorpha spp.	16.6 (µM)	V_{max} 129.4 (µmol $g_{dw}^{-1}h^{-1}$)	Harlin (1978)
Phormidium laminosum	4.5 (µM)	33.4 (µg Chl^{-1} h^{-1})	Garbisu *et al*. (1992)
Phormidium laminosum (Immobilized)	62.0 (µM)	38.6 (µg Chl^{-1} h^{-1})	Garbisu *et al*. (1992)
Macrocystis pyrifera	-	9-44 (mM cm^{-2} h^{-1})	Wheeler (1980)
Macrocystis sp.	-	0.229 (µM gFW^{-1} h^{-1})	Neushul *et al* (1992)
Enteromorpha prolifera	2.3-13.3 (µM)	V_{max} 75-169 (µmol $g_{dw}^{-1}h^{-1}$)	O'Brien and Wheeler (1987)
Ulva rigida	20-33 (µM)	V_{max} 60-90 (µmol $g_{dw}^{-1}h^{-1}$)	Lavery and McComb (1991)
Gracilaria foliifera	2.5 ± 0.5 (µM)	V_{max} 9.7 (µmol $g_{dw}^{-1}h^{-1}$)	D'Elia and DeBoer (1978)
Chordaria flagelliformis	5.9 ± 1.2 (µM)	V_{max} 8.8 (µmol $g_{dw}^{-1}h^{-1}$)	Probyn (1984)
Spirulina platensis	**450-1170 (µM) (6.3-16.44 mg NO_3-N L^{-1})**	**V_{max} 0.21-0.73 (mg NO_3-N. mgChl-a^{-1} h^{-1})**	**This study**

In natural condition, algae with the higher maximum nitrogen uptake rates (high V_{max} with low K_m) have an advantage over other algae with lower uptake rates, especially under nitrogen limitation. For example, competition between the green microalga *Scenedesmus quadricauda* and the blue-green microalga *Microcystis novacekii* was a result of different nutrient uptake rate where *M. novacekii* which had lower K_m was become a dominant species in most situation. On the other hand, *S. quadricauda* that had higher K_m became competitively superior only in the large-pulse, low-frequency nutrient supply mode (Watanabe and Miyazaki, 1996).

Ammonium uptake of *S. platensis* was the most important character to perform nitrogen treatment in the co-cultured of algae and shrimp in this study. During shrimp culture, shrimp excretion and decomposition of uneaten feed mostly produce nitrogen waste in ammonia form. Hence, the proposed technique of using *S. platensis* to treat nitrogen waste in the tank is to take up ammonia rather than nitrate. This experiment only revealed the basic knowledge of ammonium and nitrate uptake in such environmental controlled laboratory condition. Since nitrogen uptake is a process that relied on many physiological property of the cells as well as surrounding environment parameters, V_{max} and K_m derived from this experiment, however, was only the rough numbers and cannot be directly used for balancing nitrogen in aquaculture systems. Moreover, in order to use *Spirulina* for water quality control in aquaculture tank, many factors were still unknown and need to be mentioned here. These factors included the appropriate amount of *Spirulina* that should balance with nitrogen waste produced from the culture system, competitiveness between *Spirulina* and other contaminated

microalgae and algal physiology especially photosynthesis activity of *Spirulina* that enhancing nitrogen uptake rate. As low K_m and high V_{max} values may not be only the significantly advantageous for microalgal competition in all conditions, other factors such as vertical migration cycle, intracellular nutrient storage and dark assimilation are also need to be concerned (Lieberman *et al*., 1994).

3.4 CONCLUSION

This experiment involved the study on effect of salinity (0, 15 and 30 psu) on ammonium and nitrate uptake kinetics of *Spirulina* in laboratory conditions. This results could be concluded as following:

1 Ammonium and nitrate disappearance from the medium, as a result of *Spirulina* uptake, was rapidly during the first hour. Thereafter, the uptake rates were similarly declined in all treatments.

2 In this study, high salinity (15 and 30 psu) had strong affect to nitrogen uptake rate in both ammonium and nitrate forms but salinity seemed to have more affect to nitrate uptake than ammonium uptake.

3 The K_m and V_{max} constants of ammonium uptake rate by *Spirulina* cultured at 0, 15, and 30 psu were 5.470, 4.544, 3.717 mg NH_4-N L^{-1} and 0.320, 0.200, 0.120 mg-NH_4-N mgChl-a^{-1} h^{-1}, respectively.

4 The K_m and V_{max} constants of nitrate uptake rate by *Spirulina* cultured at 0, 15, and 30 psu were 16.444, 5.342, 6.293 mg NO_3-N L^{-1} and 0.732, 0.313, 0.206 mg-NO_3-N mgChl-a^{-1} h^{-1}, respectively.

CHAPTER IV

WATER QUALITY CONTROL USING *S. platensis* IN SHRIMP CULTURE TANKS

4.1 INTRODUCTION

Intensive shrimp culture in Thailand uses 30-57% protein feed with high feeding rates. Organic nitrogen waste from uneaten feed and shrimp excretions decomposes into toxic inorganic nitrogen compounds, including ammonia (NH_3 or NH_4^+) and nitrite (NO_2^-). With aerobic conditions, ammonia and nitrite are converted into relatively non-toxic nitrate (NO_3^-), but high nitrate concentrations can stress shrimp. Water exchange is therefore still recommended, especially when nitrate is ≥50 mg NO_3-N L^{-1} (Hart and O'Sullivan, 1993).

Nitrate is not easily removed by conventional water treatment. Biological nitrate removal using aerobic microalgae offers some advantage over anaerobic, microbial denitrification since both ammonia and nitrate nitrogen are readily removed and the process is less complicated (Vílchez *et al*., 1997). Microalgae play a dominant role in stabilizing earthen pond water quality. Aquacultural water treatment by macroalgae, such as *Gracilaria,Ulva, Enteromorpha* and microalgae has been used in aquaculture (Marinho-Soriano *et al*., 2002, Nelson *et al*., 2001 and Turker *et al*., 2003). Such systems are based on the concept that excretions of one organism provide the nutrients for other organisms in the pond.(Buschmann *et al*., 1996).

However, the main disadvantage of using microalgae is that algal cells are not easily removed from the culture system. If algal cells are not removed, nitrogen compounds are released back to the water. Moreover, high microalgae concentrations can cause dissolved oxygen depletions during the night due to high respiration rates. Microalgal immobilization has been used for wastewater treatment in whatever entrapment (Vílchez and Vega, 1994; Kaya *et al*., 1995; Chen, 2001) or attachment conditions (Garbisu *et al*., 1991; Gil and Serra, 1993), but these techniques are expensive and not practical on large scales with aquaculture ponds. Moreover, immobilization using carrageenan or alginate can be easily dissolved in seawater which contains salts and other ions such as phosphate and EDTA (Brodelius and Vandamme, 1987).

Ideally, microalga used with integrated aquaculture systems should have all of the following characteristics: [1] algal cells must be harvested by simple filtration; [2] easy to mass culture; [3] tolerate wide salinity range; and [4] algae are a valuable by product. With this present study, *Spirulina platensis* was chosen for nitrogen removal in shrimp culture tanks since it meets all the above requirements. *S. platensis* grew well in seawater (5-30 psu salinity) and was easily removed by filtration through 22 μm mesh net. This allowed semi-continuous harvest of *S. platensis*. Although *Spirulina* is one of the most widely studied microalgae, especially for wastewater treatment, most studies are with algal monoculture in high rate algal pond system (Tanticharoen *et al*., 1993; Phang *et al*., 2000), not with integrated systems containing both algae and an animal crop. This present research evaluated the efficacy of integrated algae-shrimp culture where *S. platensis* was used for water quality control with semi-continuous algae harvest.

4.2 MATERIALS AND METHODS

4.2.1 S. platensis culture

Stock culture of *S. platensis* was obtained from the Institute of Food Research and Product Development, Kasetsart University, Thailand and maintained in 30 psu (~0.5 M NaCl) Zarrouk medium (Zarrouk, 1966) under 100 μmol photon m^{-2} s^{-1} illumination at 25-28°C. Working algal culture were prepared by transferring stock algal culture into 2L Erlenmeyer flasks with air injection. When cultures reached mid-logarithmic growth phase, they were concentrated by filtering through 22 μm nylon net and washed with fresh Zarrouk medium without nitrate, then added to shrimp culture tanks.

4.2.2 Shrimp culture condition

Black tiger shrimp were collected from earthen ponds in Pathum Thani Province and transferred to a concrete holding tank at Marine Biotechnology Research Unit, Chulalongkorn University, Bangkok for 30 days before starting the trials. All trials were performed in 0.3x0.6x0.3 m^3 glass aquariums containing 30 L of seawater (30 psu) under semi-transparent roof **(Fig. 4.1 and 4.2)**. Aeration was provided by air stones and freshwater (0 psu) was added daily to compensate for evaporation. Feces, uneaten feed and dead shrimp were removed daily by siphon, and water was returned to the same tank. Shrimps in each tank were fed three times daily with commercial shrimp feed (36.5% crude protein) at about 4% of body weight. Shrimp survival and growth were measured

every 15 days. Weights were measured to 0.01 g. All treatments had three replicates, and trials were completely randomized.

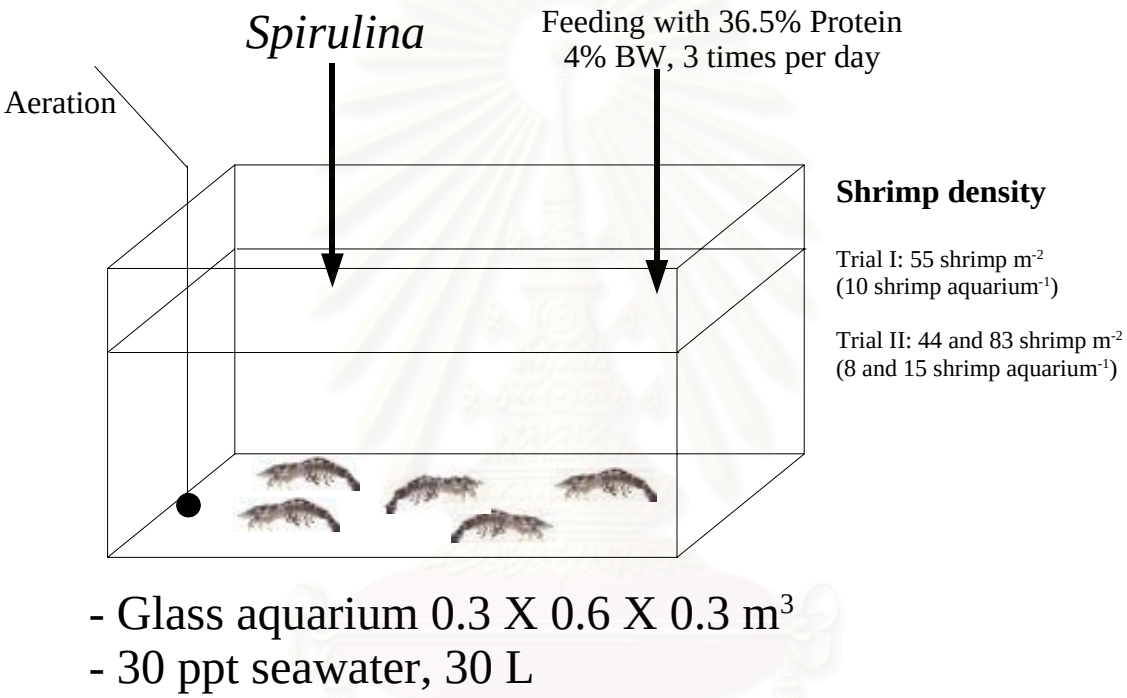

Figure 4.1 Diagram of the shrimp rearing unit used in Trial I and II.

Figure 4.2 Photograph of the shrimp rearing units used in Trial I and II.

4.2.3 Semi-continuous harvesting of S. platensis from shrimp tanks

S. platensis densities were measured using chlorophyll-*a* (Chl-*a*) concentration and trichomes counts. Trichomes were counted using a haemacytometer with light microscope, while at the same time observating for other algal contamination. Chl-*a* analysis was measured by centrifuging (5,000 rpm for 5 minutes) water samples. Thereafter, algal pellets were extracted in methanol and light absorption measured at 630, 647, and 665 nm (Bennet and Bogorad, 1972; Parson *et al*., 1989). With semi-continuous harvesting treatments, Chl-*a* in all aquaria was maintained at 0.02-0.04 mg Chl-*a* L^{-1} every two days by filtering appropriate water volumes from the aquaria through 22 μm net, then returning water to the same aquaria **(Fig. 4.3)**.

Figure 4.3 Photograph of the *S. platensis* harvested from the aquaria through 22 μm net, then returning water to the same aquaria.

4.2.4 Water quality analysis

Inorganic nitrogen compounds (NH_4-N, NO_2-N and NO_3-N) in water were measured every two days according to Parsons *et al*. (1989). pH, temperature and salinity were also monitored. Freshwater (0 psu) was added to compensate for evaporative water losses.

4.2.5 Trial I: Effect of semi-continuous harvesting of S. platensis on inorganic nitrogen in shrimp culture tanks

Shrimp were cultured at 10 per aquarium (55 shrimp m^{-2}) with three treatments and three replicates per treatment; shrimp cultured without *S. platensis* (control), shrimp cultured with un-harvested *S. platensis* (treatment 1), and shrimp cultured with semi-continuous harvest of *S. platensis* (treatment 2). Average initial shrimp weight was 4.6 g. This trial lasted 45 days.

4.2.6 Trial II: Effect of shrimp densities and semi-continuous harvest of S. platensis on inorganic nitrogen in culture tanks

Shrimp were cultured at two densities of 8 and 15 shrimp per tank (44 and 83 shrimp m^{-2}), designated low and high density respectively. Average initial shrimp weight was 3.3 g. There were four treatments with three replicates per treatment; low shrimp density without *S. platensis* (control), low shrimp density with semi-continuous *S. platensis* harvest (treatment 1), high shrimp density without *S. platensis* (control 2) and

high shrimp density with semi-continuous *S. platensis* harvest (treatment 2). This trial lasted 57 days.

4.3 RESULTS AND DISCUSSION

4.3.1 Trial I: Effect of semi-continuous harvesting of S. platensis on inorganic nitrogen in shrimp culture tanks

When concentrated *S. platensis* cells were first added to shrimp culture tanks (treatment 1 and 2), ammonium, nitrite and nitrate concentrations were greater in the treatment groups than in the control **(Fig. 4.4)**. This was due to nitrogen contamination from incompleted washed cells cultured using high nitrate Zarrouk medium. During day 1 to 7, nitrogen concentrations decreased in both treatments regulated by *S. platensis*, while nitrogen increased in the control. All nitrogen compounds in the controls increased throughout the trial with nitrate of >16 mg NO_3-N L^{-1} and ammonium and nitrite of nearly 0.5 mg-N L^{-1}.

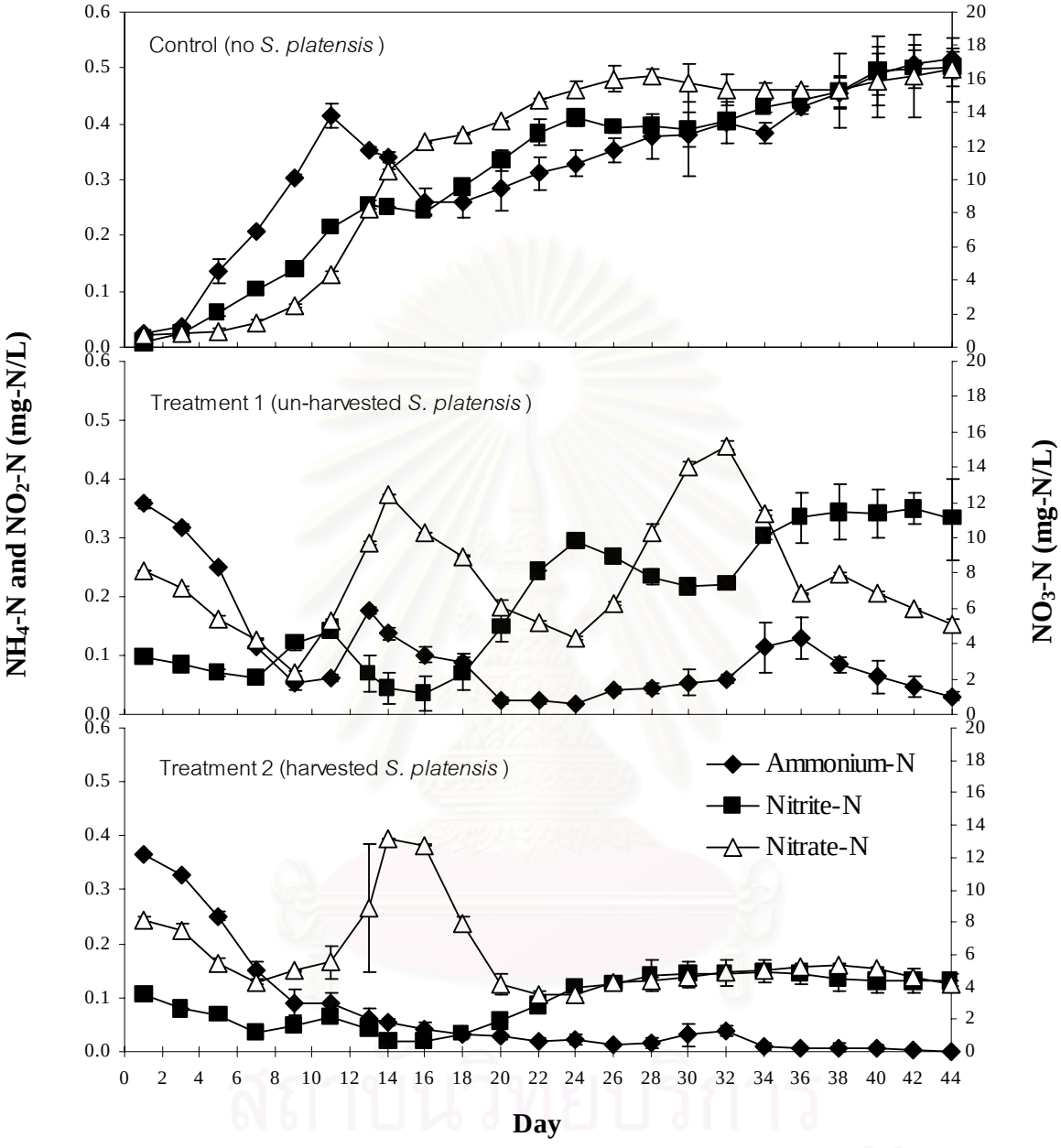

Figure 4.4 Ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N) concentrations in control tanks (shrimp without *S. platensis*), treatment1 (shrimp without *S. platensis* harvest) and treatment 2 (shrimp with semi-continuous harvest of *S. platensis*) during Trial I. Initial shrimp densities were 55 prawn m^{-2}. Error bars indicate standard deviation with *n*=3.

Chlorophyll-*a* concentration were always less than 0.02 mg L^{-1} in trial I controls, while treatment 1 and 2, Chl-*a* average 0.01 to 0.13 mg L^{-1} **(Fig. 4.5)**.

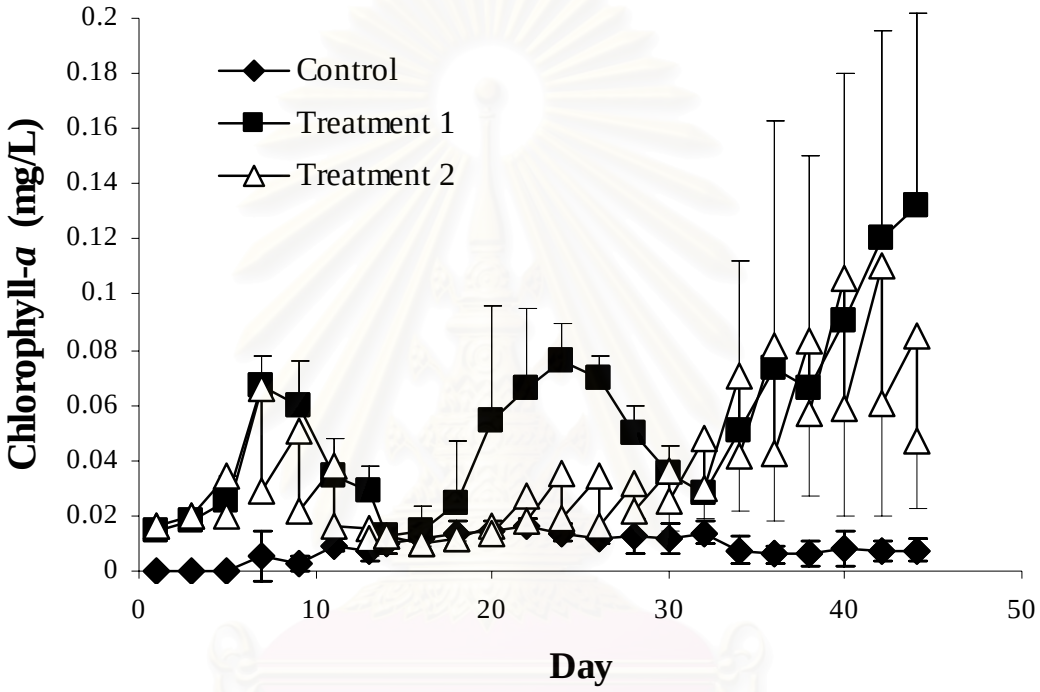

Figure 4.5 Chlorophyll-*a* concentrations in shrimp culture tanks; control (shrimp without *S. platensis*), treatment 1 (shrimp with *S. platensis*, no harvesting), and treatment 2 (shrimp with semi-continuous harvest of *S. platensis*) during Trial I. Error bars indicate standard deviation with *n*=3. For treatment 2, error bars were from chlorophyll-*a* data before harvesting.

Microscopic observation revealed low number of benthic and planktonic diatoms in both controls and treatments, but there were never abundant in any tank. Cloudy weather during days 11-20 resulted in reduced *S. platensis* growth during this time in treatments 1 and 2. *S. platensis* growth increased after day 20. Trichome number and Chl-*a* were positively correlated, with R^2=0.607 (P<0.001) in treatment 1 and 2. There was more variability with greater trichome number **(Fig. 4.6)**. This relationship further indicates that *S. platensis* was the dominant alga in those treatments. Value for pH ranged from 7.5 to 8.5, while temperatures were 28 to 31°C. Salinities averaged 30 psu (29 to 31 psu range), while alkalinity ranged from 120 to 150 mg L^{-1}. There were no significant differences (P>0.05) between those water quality parameters for the treatments and controls.

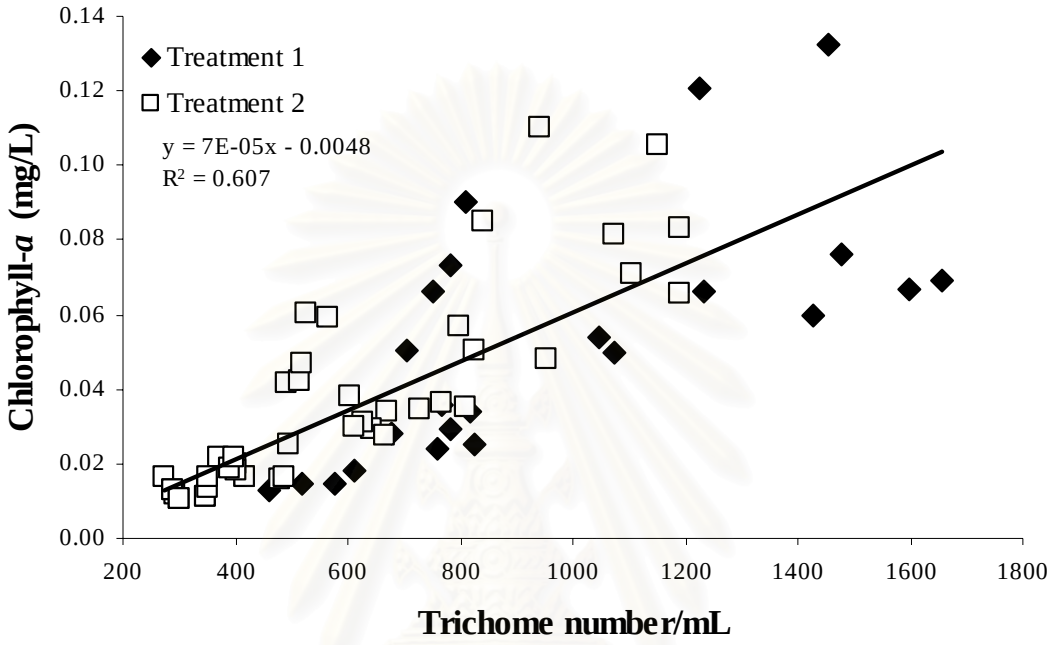

Figure 4.6 Relationship between *Spirulina* trichome numbers and chlorophyll-*a* concentrations in shrimp culure tanks during Trial 1. Values are shown for treatment 1 (shrimp with *S. platensis*, no harvesting) and treatment 2 (shrimp with semi-continuous harvest of *S. platensis*). Regression line is for both treatments.

Nitrogen compounds fluctuated the most in trial I, treatment 1 (Fig. 4.4). Nitrate decreased from 8 to 3 mg NO_3-N L^{-1} during days 1-10, then increased to >12 by day 13 during reduced algal growth caused by cloudy weather. Nitrate increased again on day 31 with >15 mg-N L^{-1} before decreased to <6 by day 44. Nitrite concentrations ranged from <0.1 to >0.3 mg NO_2-N L^{-1} and cycled out of synchronize with nitrate. Ammonium concentration were mostly <0.1 mg NH_4-N L^{-1}.

With semi-continuous *S. platensis* harvest (trial I, treatment 2), nitrogen compounds were consistently low during most of the culture period (Fig. 4.4). Only nitrate increased between days 10 and 16 due to cloudy weather reduced algal growth. During days 34-44, ammonium was nearly un-detectable.

Average weights of shrimp on days 30 and 45 of the two treatment groups were not significantly different, but both treatments had significantly greater weights than the control group (P<0.05; **Fig. 4.7)**. Shrimp survivals averaged 53.3±11.5, 43.3±5.8 and 60±10% on day 45 for control, treatment 1 and treatment 2 respectively. Average survival rate were not significantly different (P>0.05; **Fig. 4.8;** Appendix D).

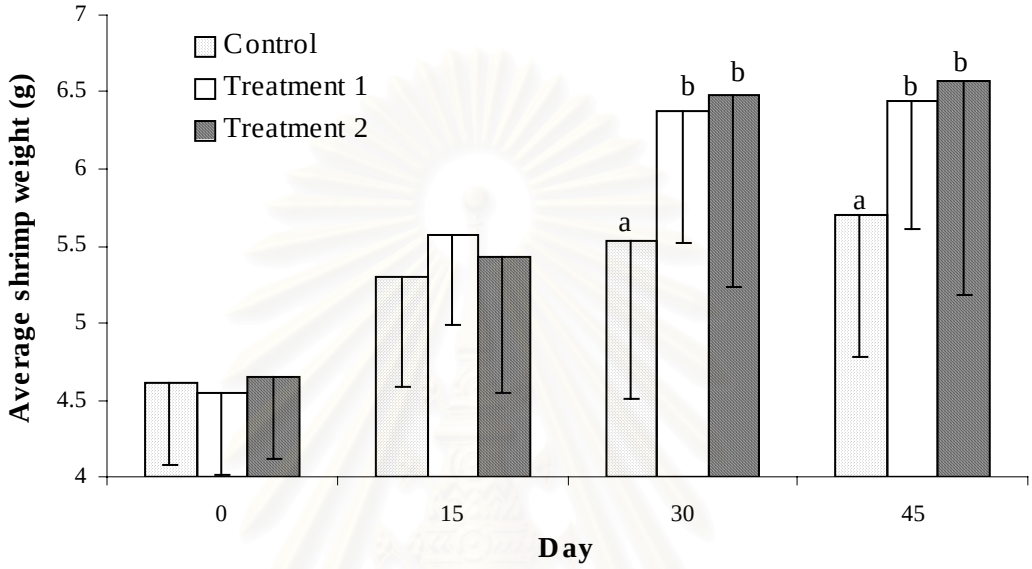

Figure 4.7 Average shrimp weights (g) in Trial I of control (shrimp without *S. platensis*), treatment 1 (shrimp with un-harvested *S. platensis*), and treatment 2 (shrimp with semi-continuous harvest *S. platensis*). Error bars represent standard deviation (n=30) while a or b indicate significant differences ($P \leq 0.05$).

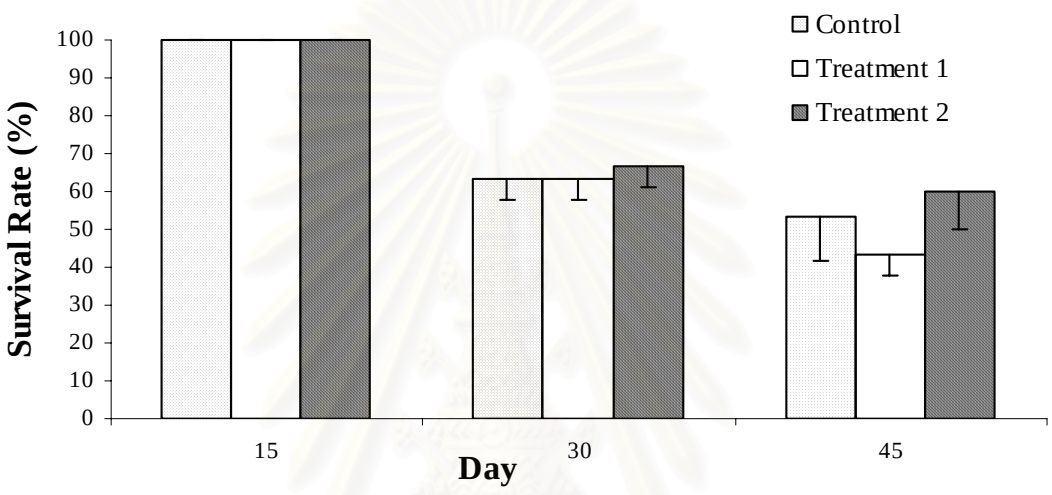

Figure 4.8 Average shrimp survival rate (%) in Trial I of control (shrimp without *S. platensis*), treatment 1 (shrimp with un-harvested *S. platensis*), and treatment 2 (shrimp with semi-continuous harvest *S. platensis*). Error bars represent standard deviation (n=30) while a or b indicate significant differences ($P \leq 0.05$).

4.3.2 Trial II: Effect of shrimp densities and semi-continuous harvest of S. platensis on inorganic nitrogen in culture tanks

Water quality in trial II can be grouped as shrimp cultured with *S. platensis* (treatment 1 and 2), and shrimp cultured without *S. platensis* (control 1 and 2). In treatment 1 and 2, semi-continuous harvesting of , *platensis* provided excellent water quality control with both low and high shrimp densities **(Fig. 4.9)**. On the other hand, ammonium, nitrite and nitrate were elevated in both control groups without *S. platensis*. Chl-*a* concentrations in both control groups were similar and much lower than treatment groups **(Fig. 4.10)**. In control 2, ammonium and nitrite reached 0.5 mg-N L^{-1} while nitrate increased to >20 mg NO_3-N L^{-1} by day 60. Continuous exposure to these nitrogen concentrations could reduce survival.

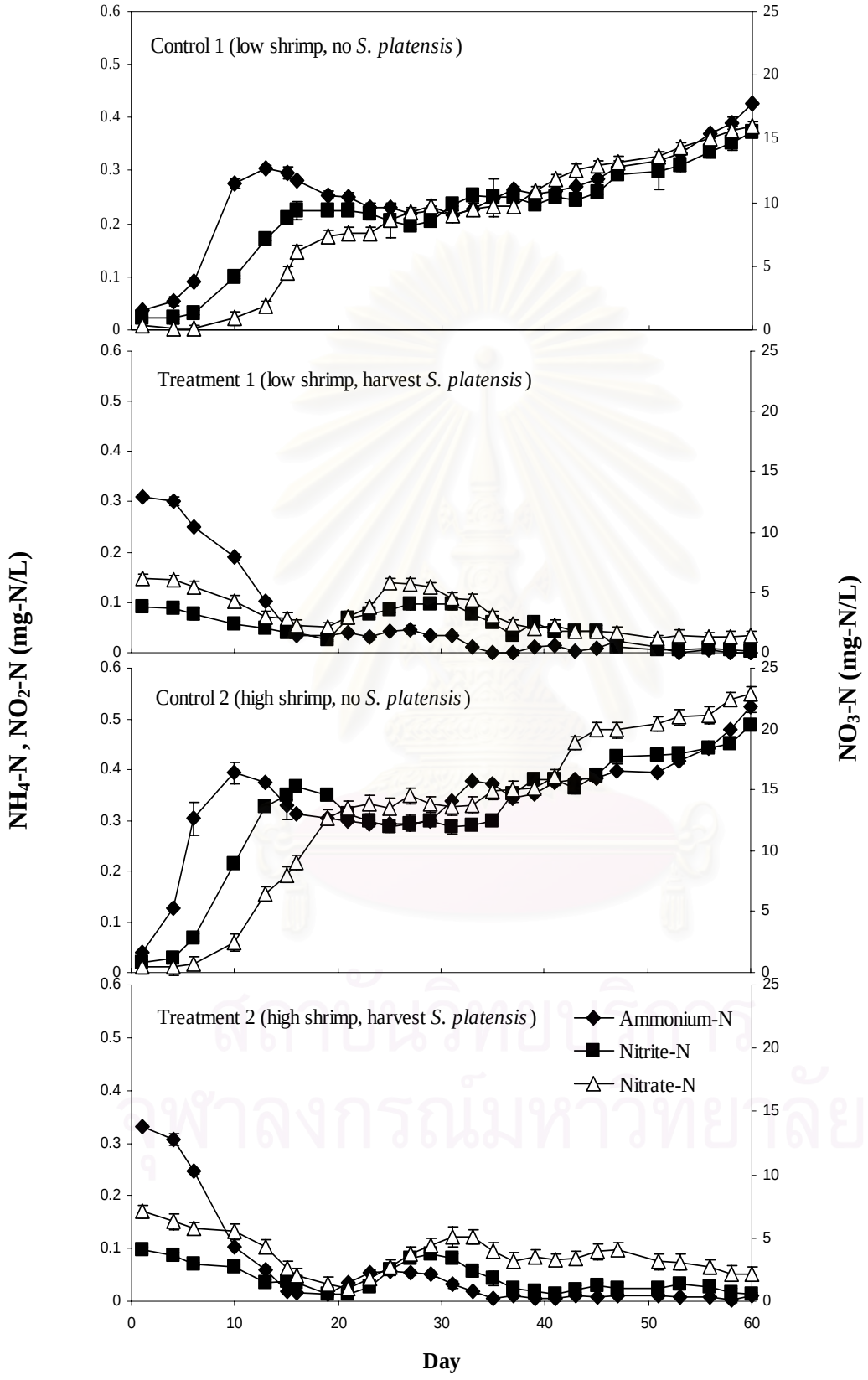
Control 1 (low shrimp, no S. platensis)
Treatment 1 (low shrimp, harvest S. platensis)
Control 2 (high shrimp, no S. platensis)
Treatment 2 (high shrimp, harvest S. platensis)
Ammonium-N
Nitrite-N
Nitrate-N
NH4-N , NO2-N (mg-N/L)
NO3-N (mg-N/L)
Day
0
10
20
30
40
50
60
0.1
0.2
0.3
0.4
0.5
0.6
5
15
25

Figure 4.9 Ammonium, nitrite and nitrate concentrations during Trial II for control 1 (low-density shrimp without *S. platensis*), treatment 1 (low-density shrimp with semi-continuous harvesting of *S. platensis*), control 2 (high-density shrimp without *S. platensis*) and treatment 2 (high-density shrimp with semi-continuous harvesting of *S. platensis*). Shrimp density was 44 or 83 m^{-2} for low and high density respectively. Error bars indicate standard deviation with n=3.

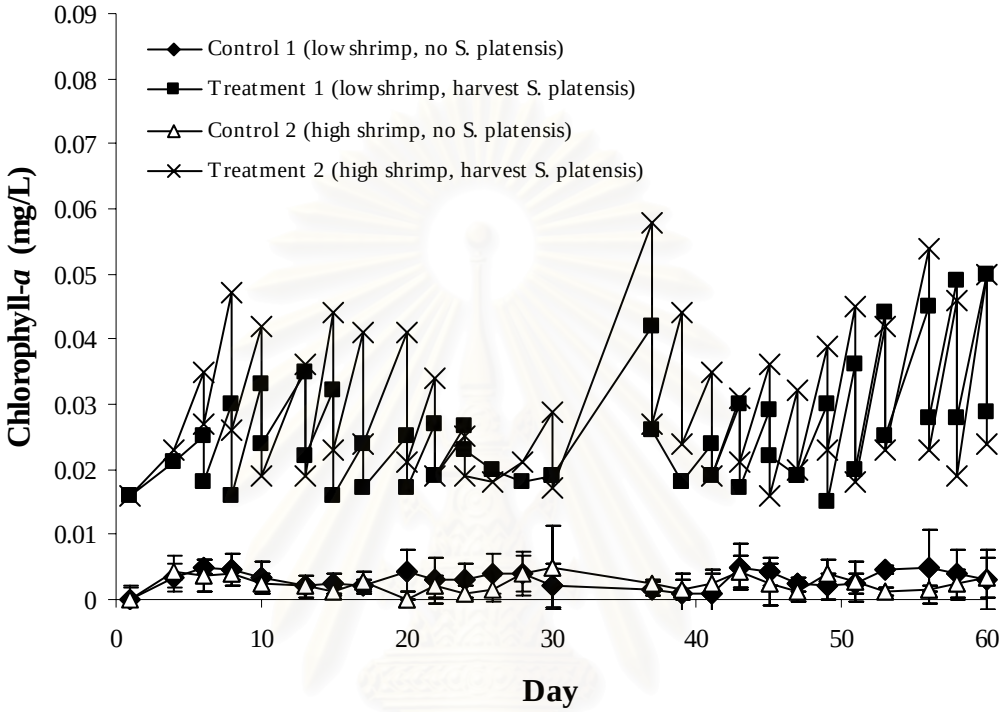

Figure 4.10 Chlorophyll-*a* concentrations in Trial II control 1, treatment1, control 2, and treatment 2. Shrimp densities were 44 or 83 m^{-2} for low and high density respectively. Error bars of control groups indicate standard deviation with *n*=3. For treatment 1 and 2, only average data of 3 replicates were shown.

Semi-continuous harvest of *S. platensis* maintained Chl-*a* between 0.02-0.05 mg L^{-1} and maintained cells in exponential growth phase throughout trial II. Decrease in inorganic nitrogen during the first 10 days of trial II was similar to the decrease in trial I. During trial II average shrimp weights were not significantly difference ($P>0.05$) between groups **(Fig. 4.11)**. However, analysis of covariance (ANCOVA) showed that growth rate of shrimps in treatment 1 was significantly ($P<0.05$) higher than in control 2. Moreover, higher density shrimp culture without *S. platensis* (control 2) had significantly ($P<0.05$) lower survival rate compared with other treatments **(Fig. 4.12)**.

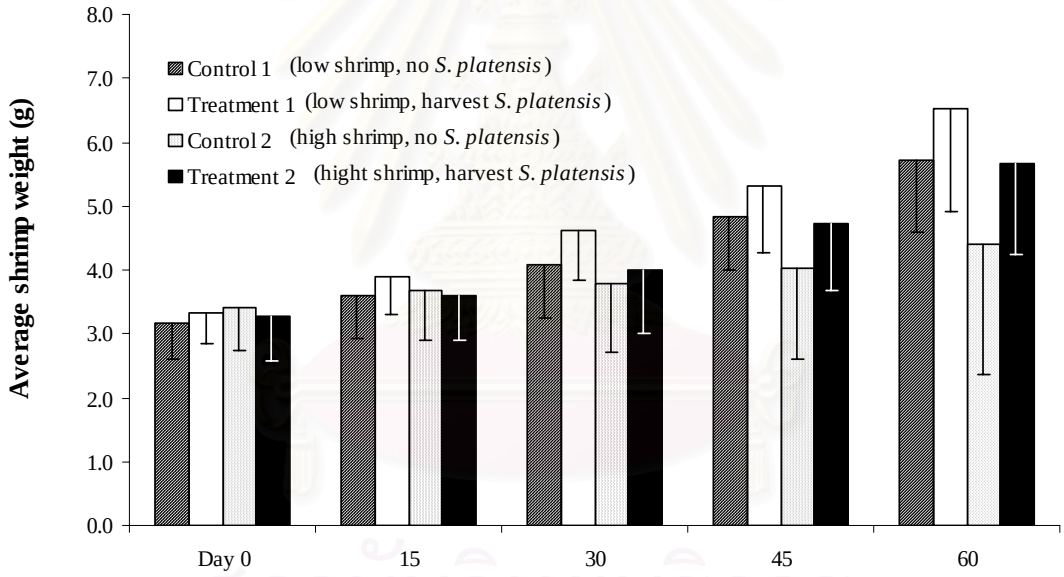

Figure 4.11 Average shrimp weights (g) in Trial II of control 1 (low shrimp density, no *S. platensis*), treatment 1 (low shrimp density, harvested *S. platensis*), control 2 (high shrimp density, no *S. platensis*) and treatment 2 (high shrimp density, harvest *S. platensis*).

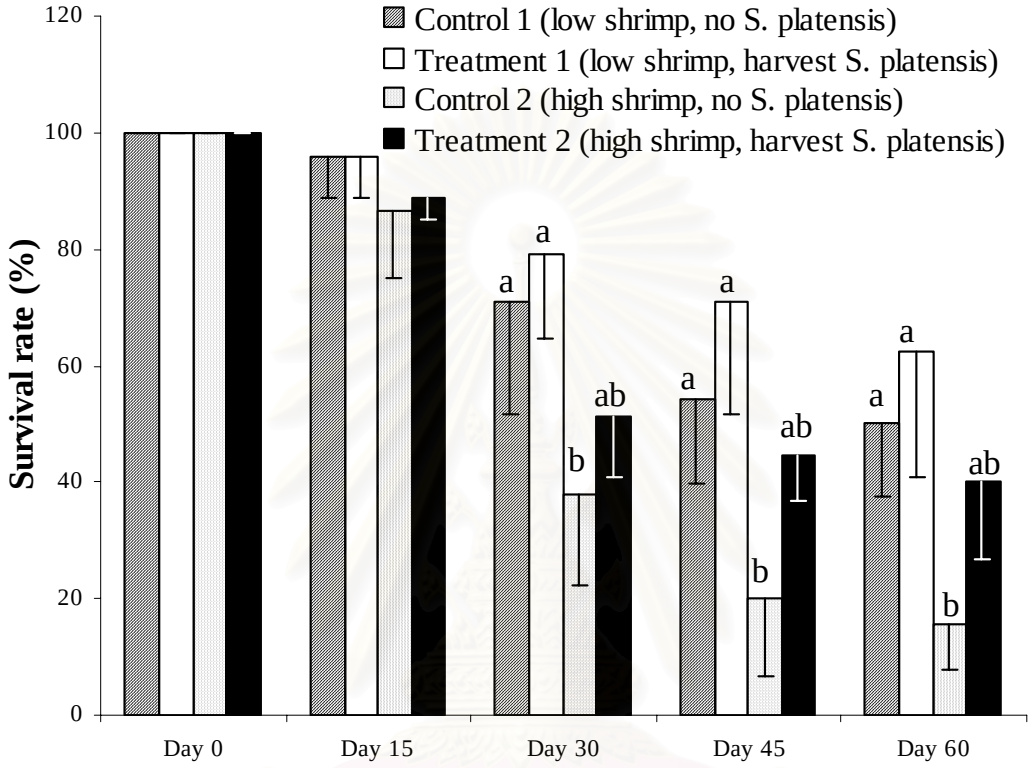

Figure 4.12 Shrimp survival rate (%) with and without co-culture with *S. platensis*. Shrimp densities were 44 or 83 m^{-2} with low and high densities respectively. Error bars represent standard deviation (n=3), while a or b indicates statistical significant differences ($P \leq 0.05$).

4.3.3 DISCUSSION

With intensive shrimp culture systems, low phytoplankton concentrations are an unlikely event since there is ample of nutrients from feed addition and shrimp excretion to sustain dense algal growths. In Thailand, most shrimp farmers believed that desirable "brownish-green water color" from algae will increase shrimp growth and survival. This color is usually due to diatoms and green algae. Thai farmer practice similarity concept like Costa-Pierce (2002), the integration of agriculture and aquaculture on farm in Asia created definable aquaculture ecosystem as ecological aquaculture model, which closely resembled natural ecosystems, with their own structure, closely coupled natrient-recycling pathways and ecological management. Many farmers also add fertilizer to the ponds (Boyd, 1995). That sometimes causes red tides, or blooms of uncontrolled algal species such as diatom, dinoflagellates or blue-green algae in the ponds. Attempt to add bulk cultured microalgae to earthern shrimp culture ponds have been attempted during the past 10 years by both farmers and researchers in Thailand (personal communications). However, phytoplankton, which normally have a life spans of 1 to 2 weeks (Boyd and Musig, 1992), soon dies and decomposes releasing nitrogen into the water.

The concept of integrating *S. platensis* with shrimp differs from other attempts of algal management since we controled algal cells at a desired concentration. This study was designed to maintain low cell concentration of 0.02 mg Chl-*a* L^{-1}. At this concentration, water colour in our tanks was not the dark-green typical of *S. platensis* production ponds. Semi-continuous harvesting also maintained algae in exponential

growth, never in stationary and death phases. This maintained healthy *S. platensis* with rapid nutrient uptake.

Typically, 70-80% of nitrogen from applied feed remains in an aquaculture pond (Avnimelech and Lacher, 1979 cited in Zur, 1981; Boyd, 1985; Funge-Smith and Briggs, 1998; Thakur and Lin, 2003). Most nitrogen loss from the pond is through denitrification from anoxic bottom sediment. However, anoxic condition or even low dissolved oxygen at the bottom should be avoided since it can negatively effects shrimp survival (Martin *et al*., 1998). This study indicates a possibility for integrated culture of shrimp and *S. platensis*. This findings clearly show that *S. platensis* reduced inorganic nitrogen and resulted in excellent water quality for shrimp, provided that some algal biomass was continuously removed. Since the experiment were in glass aquariums, nitrogen cycling was more easily traced. Adaptation of this process to large earthen ponds may require a special designed algal harvesting system. Photoinhibition, which is the inhibition of algal photosynthesis by strong sunlight, must be considered and this can be avoided by adding shade or roof. Normally, dense *S. platensis* culture could loss productivity up to 30% by photoinhibition (Vonshak *et al*., 1988). This should be more affect to algal cells in our system because it is maintained at the lower density than conventional *S. platensis* culture ponds.

It was found that algal contamination (mostly diatoms) in the tanks was not a problem although there were some contaminants and ample nutrients in the tank. Low silicate concentrations could account for this.

This integrated system of shrimp and semi-continuous harvest *S. platensis* demonstrated an excellent water quality control even at high shrimp density of 83 shrimp m^{-2}. Conventional water quality control in intensive shrimp culture systems is based on bacterial and is therefore size limited biofiltering. This system can be more easily expanded into large tanks or ponds for various aquatic animal culture. Water quality in the shrimp tanks contain very low nitrate concentrations, and water exchange between crops was not necessary. However, more work is needed with these large culture systems. Specifically, we need to develop effective algal harvest techniques for large tanks and ponds need to be developed, and it need to determine whether *S. platensis* (or another desirable alga) can be maintained as the dominant alga throughout the culture cycle.

4.4 CONCLUSION

A cyanobacterium (*S. platensis*) was co-cultured with black tiger shrimp (*P. monodon*) for water quality controly. The results could be concluded as following:

1 Integrated shrimp culture with *Spirulina* in closed culture system can maintain acceptable water quality for shrimp growth. While high concentration of nitrite (> 16 mg NO_2-N L^{-1}) was found in control after 44 days of culture.

2 Semi-continuous harvest of *S. platensis* resulted in significantly reduced (P<0.05) ammonium and nitrite concentration in the water without nitrate accumulation.

3 Un-harvested *Spirulina* treatment had higher nitrite and nitrate concentration than in the harvested *Spirulina* treatment, indicated that removal of *Spirulina* biomass from the tank resulted in nitrogen removal from the culture system.

4 For the first trial with 10 shrimps per aquarium (55 shrimps m^{-2}), co-culture of *Spirulina* with shrimp in both treatments had higher final shrimp weight than in control but the survival rate was not significantly difference.

5 Water quality in the second trial resembled the first trial since *Spirulina* could successfully reduce ammonium and nitrite concentration without nitrate accumulation.

6 For the second trial with 8 or 15 shrimps per aquarium (44 or 83 shrimps m^{-2}), significantly higher survival rate was obtained in the treatments with *Spirulina*.

CHAPTER V

WATER QUALITY AND NUTRIENTS BUDGET IN INTEGRATED SHRIMP CULTURE WITH *S. platensis* UNDER OUTDOOR CONDITION

5.1 INTRODUCTION

The closed recirculating system for fish and shrimp culture has seen a rapid growth in recent years. Most of the systems used in the indoor tanks consist of three main components *i.e.* (1) sediment removal unit with sedimentation or filtration apparatus, (2) biofilter unit often with nitrofication biofilter but rarely with denitrification, and (3) water sterilization unit by either UV or Ozone treatment. Water quality in outdoor ponds, on the other hand, depends on both physical and biological factors which mostly uncontrollable. The only water quality improvement techniques for outdoor pond are aeration with paddle wheels and water exchange.

In general, the water treatment system for indoor closed recirculating system is usually not suitable for the outdoor pond because light will interfere the system by inducing growth of phytoplankton and reducing activity of nitrifying bacteria. In the previous chapter, co-culture of shrimp with *S. platensis* showed the high potential of water quality control through the microalgal harvest. Shrimp-*Spirulina* system proposed in this study requires light for *Spirulina* photosynthesis, therefore it must be used under an outdoor condition in the cement tank or plastic lined pond without soil bottom.

However, to apply this concept for shrimp culture in larger scale, many factors still need to be studied. One of the most important problems is how to maintain *Spirulina* the dominant species in the tank containing other phytoplankton species. High turbidity water in the tank reduces the efficiency of light utilization by *Spirulina* and make algal harvesting more complicate.

In this experiment, The shrimp-*Spirulina* system was carried out in half-ton fiberglass tanks with very high shrimp density, 120 shrimps m^{-2} bottom area, which was three times higher than conventional intensive culture. At this high density, high concentrations of nitrogen and phosphorus waste were produced and could possibly harm shrimps if there was no attempt to treat those wastes.

Shrimp culture especially black tiger shrimp, *P. monodon*, is different from many other aquatic pelagic species because it is mostly stay at the bottom of the tank or pond. Cannibalism behavior, where the newly molted or weak shrimps were eaten by other shrimps, can be a serious problem with dense culture. An artificial shelter was then provided as another treatment in the experiment. This shelter was a hiding area for shrimp and increased the bottom area of the tank.

Moreover, in order to evaluate the efficiency of algae in nutrient removal from the closed shrimp culture system under the ambient outdoor condition, nitrogen and phosphorus budget in the tank was studied. Mass balance of nitrogen and phosphorus in the aquaculture system, so call nitrogen and phosphorus budget, is the basic step for the

quantitative study of food utilization efficiency, pond fertility, water quality and processes in the sediments (Thakur and Lin (2003). This can be therefore the best procedure to evaluate the efficiency of water treatment system. Since nitrogen and phosphorus is incorporated into *Spirulina* cells, determination of nitrogen and phosphorus in *Spirulina* biomass harvested from the system will illustrated the percentage of nutrients removal by the algae not by other processes such as denitrification, volatilization or water drainage. The present study aimed to investigate the efficiency of *Spirulina* for water quality control in very high shrimp culture in outdoor tanks with and without shelter using nutrients budget study.

5.2 METERIALS AND METHODS

5.2.1 Preparation of S. platensis culture

Stock culture of *S. platensis* was obtained from the Institute of Food Research and Product Development, Kasetsart University, Thailand. *S. platensis* was maintained in 30 psu (~0.5 M NaCl) Zarrouk medium (Zarrouk, 1966) under 100 μmol photon m^{-2} s^{-1} illumination at 25-28°C. Mass culture of *S. platensis* was prepared by transferring stock algal culture into 2L Erlenmeyer flasks with air bubbling. When cultures reached mid-logarithmic growth phase, they were concentrated by filtering through 22 μm nylon net and washed with fresh Zarrouk medium without nitrate, then added to shrimp culture tanks.

5.2.2 Experimental design and shrimp culture condition

In this experiment, *S. platensis* was co-cultured with shrimp in 500L fiberglass tanks (90 cm in diameter with 80 cm in height) under outdoor condition. Each tank contained 480L of 30 psu seawater with aeration through 4 air stones. Initial weight of shrimp was 4.57±1.31 g and initial density of shrimp was 72 per shrimps tank, which equal to 120 shrimps m^{-2} bottom area. This density was three times higher than that in conventional intensive shrimp culture pond. The experiment consisted of three treatments *i.e.*, shrimp culture without *S. platensis* (control), shrimp cultured with harvested *S. platensis* (treatment 1) and shrimp cultured with harvested *S. platensis* and

shelter (treatment 2). Each treatment had two replicates and the experimental plan was completely randomized design. The experiment was conducted for 57 days without water exchanged except an addition of freshwater at approximately 1L day^{-1} to compensate evaporation.

The shelters used in this experiment were made of rolled into cylinder shape plastic net with 15 cm in diameter and 60 cm in length. Each set of shelter composed of totally 12 rolled net arranged in four stacks with PVC frame to maintain cubic shape. The aim of using shelters was to provide hiding area for shrimps since shrimp density in this experiment was extremely high. Diagram and photograph of the shelter is shown in **Fig. 5.1**

Before starting experiment, water in each tank was partially sterilized by 30 minutes ozonation. Shrimps obtained from outdoor earth pond were acclimated in cement tank prior to experiment condition for 15 days before starting the experiment. During experiment, shrimp in each tank were fed three times a day with commercial shrimp pellets (36.5% crude protein) at 4% of body weight.

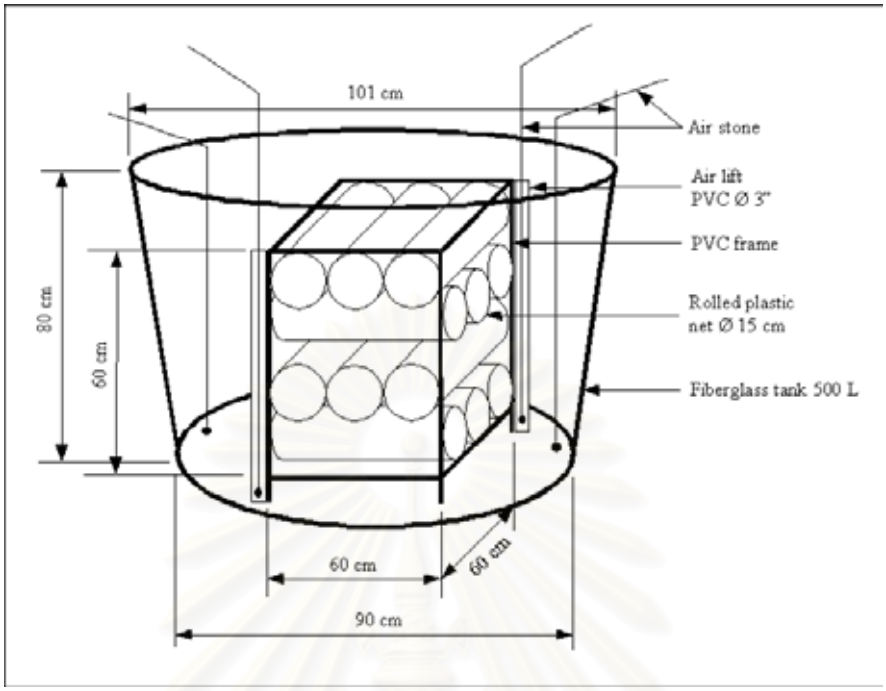

Figure 5.1 Diagram (top) and photograph (bottom) of the shrimp shelter used in this experiment. Shrimp found in the shelter were illustrated with the circles.

5.2.3 Semi-continuous harvesting of S. platensis from shrimp tanks

After adding *S. platensis* into the shrimp tank, density of *S. platensis* in all tanks was regularly monitored every two days using chlorophyll-*a* (Chl-*a*) concentration and number of trichome were counted with Sedgwick-Rafter counting chamber under light microscope. Contamination of other microalgal species was also observed during trichomes counting. With semi-continuous harvesting treatments, Chl-*a* in treatment 1 and 2 tanks were maintained at 0.02-0.04 mg Chl-*a* L^{-1} by filtering appropriate water volumes from the tank through 22 μm net, then returning water back to the same tank. Concentration of Chl-*a* was determined by filtering 10 mL of water sample through Whatman GF/C filters and following by hot methanol extraction at 70ºC for 2 minutes. Then optical density was measured spectrophotometrically at 630, 647, and 665 nm and Chl-*a* concentration was calculated as described in Bennet and Bogorad (1972) and Parson *et al.* (1989). Detail of Chl-*a* analysis is shown in Appendix B.

5.2.4 Water quality analysis

Throughout the experiment, water samples were collected every two days from the middle of the tank for water quality analysis. For nutrients analysis, water was filtered through GF/C glass fiber filter and kept in refrigerator (-20ºC). Inorganic nutrients *i.e.*, ammonium, nitrite, nitrate and phosphate, were analyzed by colorimetric methods according to Parson *et al*. (1989). Total phosphorus content was analyzed by sulfuric acid - nitric acid digestion method and followed by ascorbic acid method (Takeuchi, 1988). Water temperature and pH were monitored using automatic logging

pH meter (Hanna HI 98240). Salinity was measured using hand refractometer every two days. Alkalinity was determined by titration with 0.02N H_2SO_4 until pH reach the endpoint at pH 4.5 (APHA, 1992). Detail of all water quality analysis methods are shown in the Appendix B.

5.2.5 Growth and survival of shrimp

Shrimp growth was determined by weighting fifteen shrimps from each tank with two decimal electronic balances every two weeks. At the end of experiment, all shrimps from each tank were harvested and weighed for production evaluation then the number of shrimp was counted for survival rate calculation.

5.2.6 Determination of nitrogen and phosphorus budget

5.2.6.1 Analysis of total nitrogen and phosphorus in shrimp feeding, shrimp carcass, molt, suspended solid and algae

Shrimp feed, body (carcass), molt, settle solid or algae were dried in the hot-air oven at 105°C for 24 hours. Dry matter was then ground and homogenized with mortar and pestle. Total nitrogen content in dry matter was determined using Kjeldahl method according to standard method for food analysis (AOAC, 1980). Total phosphorus content in dry metter was analyzed by sulfuric acid - nitric acid digestion method and followed by ascorbic acid method as described in Takeuchi (1988).

5.2.6.2 Nutrient budget calculation

Evaluation of nutrient budget in the present study refers to balance and dynamics of nitrogen or phosphorus in each tank. For nitrogen budget, input and output of nitrogen compounds in feed and in *S. platensis* were determined by total nitrogen analysis using Kjeldahl method (AOAC, 1980) while inorganic nitrogen (ammonium, nitrite and nitrate) in the water was analyzed by colorimetric methods as previously described (see 5.2.4). Shrimp, *S. platensis*, total nitrogen in the water at the beginning and entirely shrimp feed used for the whole experiment were counted as sources of nitrogen input. On the other hand, nitrogen output consisted of shrimp at the end of the experiment, harvested *S. platensis*, total settle solid, other sessile macroalgae, shrimp molting and inorganic nitrogen in the water. Feeding rate and water exchanging data were also daily recorded. For phosphorus, total phosphorus was analyzed according to Takeuchi (1988) while phosphorus budget were evaluated with the same procedure as nitrogen budget.

The general balances of nutrient (nitrogen or phosphorus) in shrimp culture tank is described as the following equations (equation 1 to 3). The definition of each parameter is shown in **Table 5.1**

Nutrient input = Nutrient output…….(1)

$^{N}Shrimp_{in} + {}^{N}Feed_{in} + {}^{N}Water_{in} + {}^{N}\textit{Spirulina}_{in}$

$= {}^{N}\text{Shrimp}_{out} + {}^{N}\text{Water}_{out} + {}^{N}\textit{Spirulina}_{out} + {}^{N}\text{settle solid}_{out} + {}^{N}\text{Other algae}_{out} +$

${}^{N}\text{Molting}_{out}$ ……..(2)

${}^{N}\text{Input} - {}^{N}\text{Output} = \text{Unidentified (unaccounted) nutrient}$…… (3)

Table 5.1 Definition of parameters used for nitrogen budget calculation in this study. The same procedure was also used for phosphorus budget calculation.

Parameters	Definition
$^{N}Shrimp_{in}$	Nitrogen in shrimp at the beginning of the experiment. = total shrimp weight × 76.4% (average percentage of dry weight per fresh weight) × 11.1% (average percentage of nitrogen in dry shrimp)
$^{N}Feed_{in}$	Nitrogen in shrimp feed. = total weight of feed used throughout the experiment × 5.85% (average percentage of nitrogen in 69.18% protein feed)
$^{N}Water_{in}$	Total inorganic nitrogen in the water at the beginning of the experiment. = concentration (g-N L^{-1}) of ammonium-N + nitrite-N + nitrate-N × 480L of water in each tank
^{N}*Spirulina*$_{in}$	Nitrogen in *S. platensis* cells added into shrimp tank at the beginning of the experiment. = (((1.3477*10^{-7}) *T*N)/1000) where T = amount of trichome (trichome^{-1}), N = average percentage of nitrogen content in dry *S. platensis* as 9.03%.
$^{N}Shrimp_{out}$	Nitrogen in shrimp at the end of experiment. = [total shrimp weight × 76.4% (average percentage of dry weight per fresh weight) × 11.1% (average percentage of nitrogen in dry shrimp)]

Table 5.1

(Continued)

Parameters	Definition
$^{N}Water_{out}$	Total inorganic nitrogen in the water at the end of the experiment.
$^{N}Spirulina_{out}$	Nitrogen in *S. platensis* cells that were harvested out during the experiment plus Nitrogen in *S. platensis* cells at the end of the experiment.
$^{N}settle\ solid_{out}$	Nitrogen in settle solid found in the tank at the end of experiment. = volume of water in the tank (480L) × dry weight of settle solid × 4.359% of nitrogen content
$^{N}Other\ algae_{out}$	Nitrogen in other microalgae and sessile macroalgae found at the end of experiment. = total weight of dried sessile algae scratched from tank's wall and shelter × 3.89% of nitrogen content
$^{N}Molting_{out}$	Nitrogen in shrimp molt that were found in the tank at the end of experiment. = dry weight of shrimp molt × 5.35% of nitrogen content

5.3 RESULTS AND DISCUSSION

5.3.1 Water quality

During the first ten days of the experiment, ammonium concentration in all treatments increased up to 6-7 mg NH_4-N L^{-1} (**Fig. 5.2-5.4)**. Thereafter, ammonium was rapidly decrease to lower than 1 mg NH_4-N L^{-1} and remained constant at this concentration untill the end of the experiment. This was possibly related to an incubation time of the nitrifying bacteria rather than phytoplankton uptake. After 10 days, nitrifying bacteria could active enough for converting all ammonium to nitrite and finally nitrate. Increase of nitrite and nitrate after the decline of ammonium concentration during day 10-20 finally confirmed this hypothesis. Total inorganic nitrogen (TIN) in control, however, was consistently high while TIN in treatment 1 and 2 decreased after day 30. This suggested that nitrate in both treatments was eliminated by the algal activity.

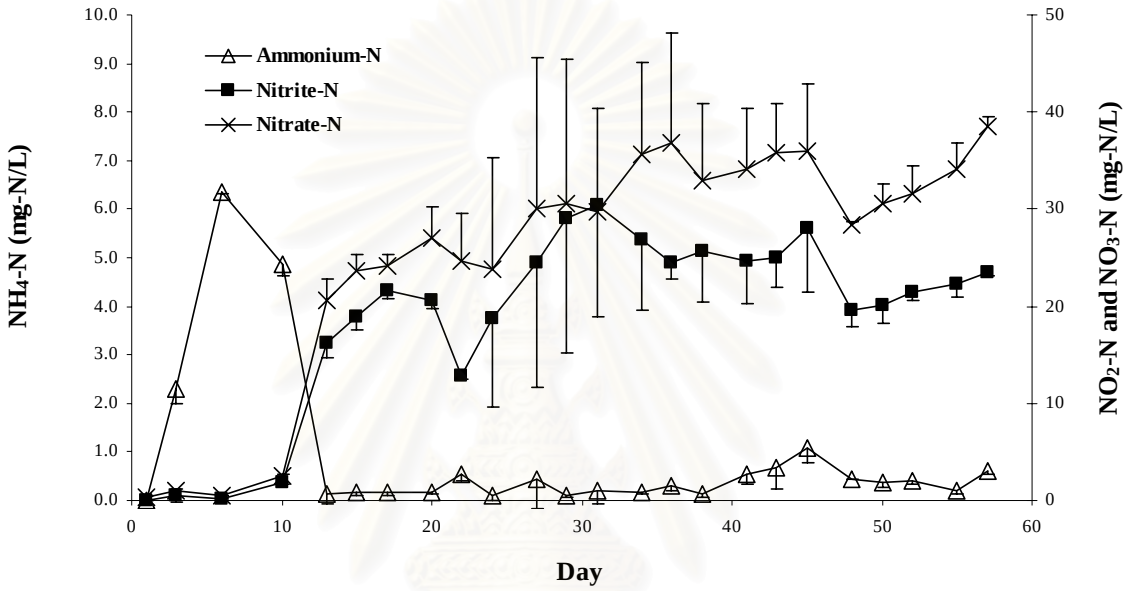

Figure 5.2 Ammonium, nitrite, and nitrate concentrations during the experiment for control (high density shrimp without *S. platensis* and shelter).

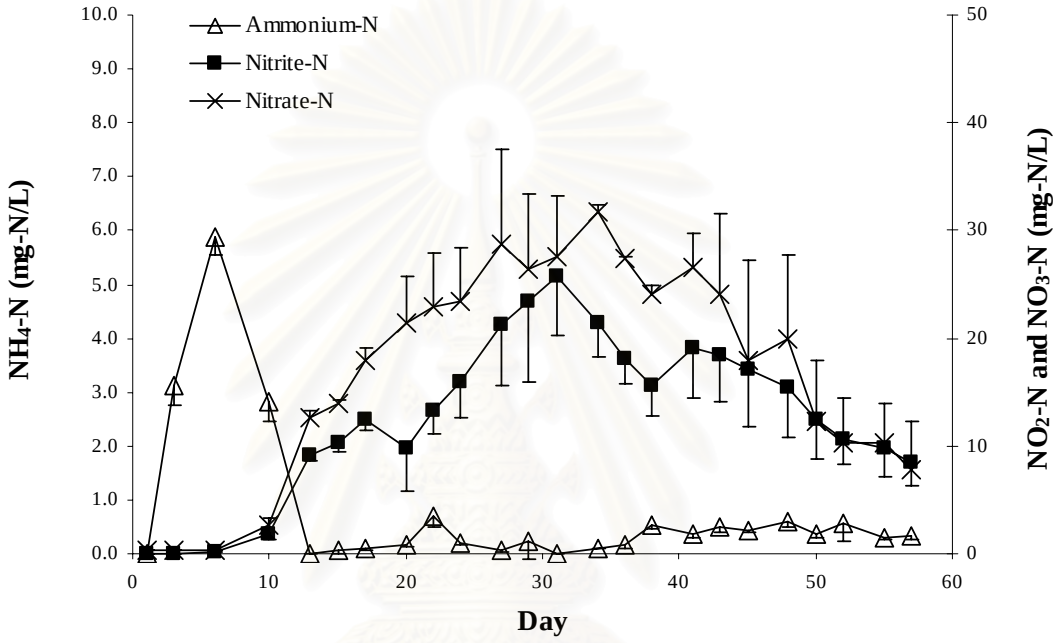

Figure 5.3 Ammonium, nitrite, and nitrate concentrations during the experiment for treatment 1 (high density shrimp with *S. platensis*).

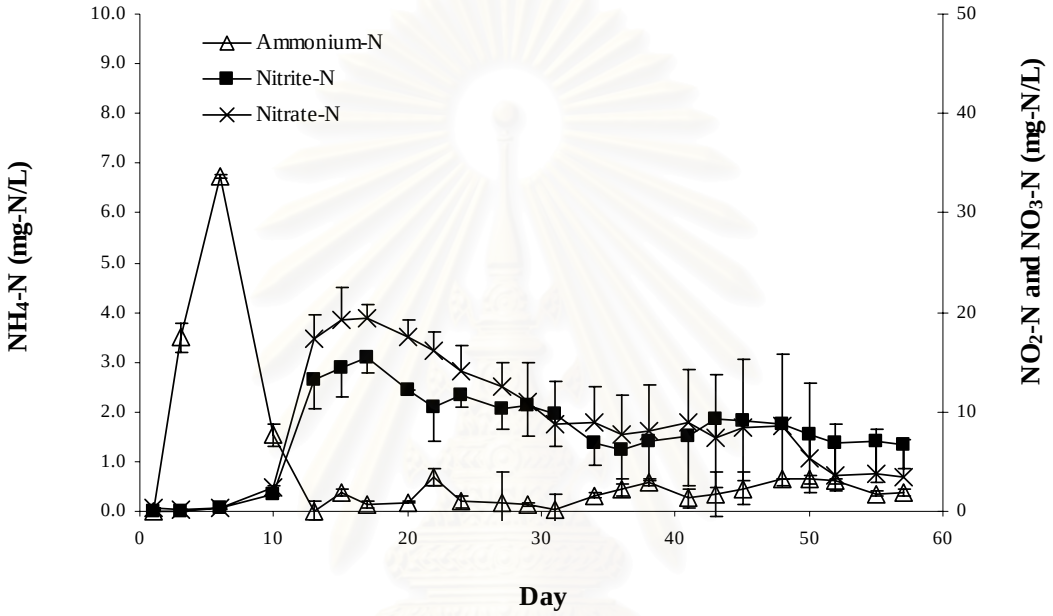

Figure 5.4 Ammonium, nitrite, and nitrate concentrations during the experiment for treatment 2 (high density shrimp with *S. platensis* and shelter).

It was found that phosphate concentration increased with time in all treatments (**Fig. 5.5**). The final phosphate concentration around 6-7 mg-P L^{-1} was very high compare with typical aquaculture ponds (Ray and Chien, 1992; Nelson *et al.*, 2001). However, harvesting of *S. platensis* in treatment 1 during day 35-40 could successfully remove some phosphate out of the system.

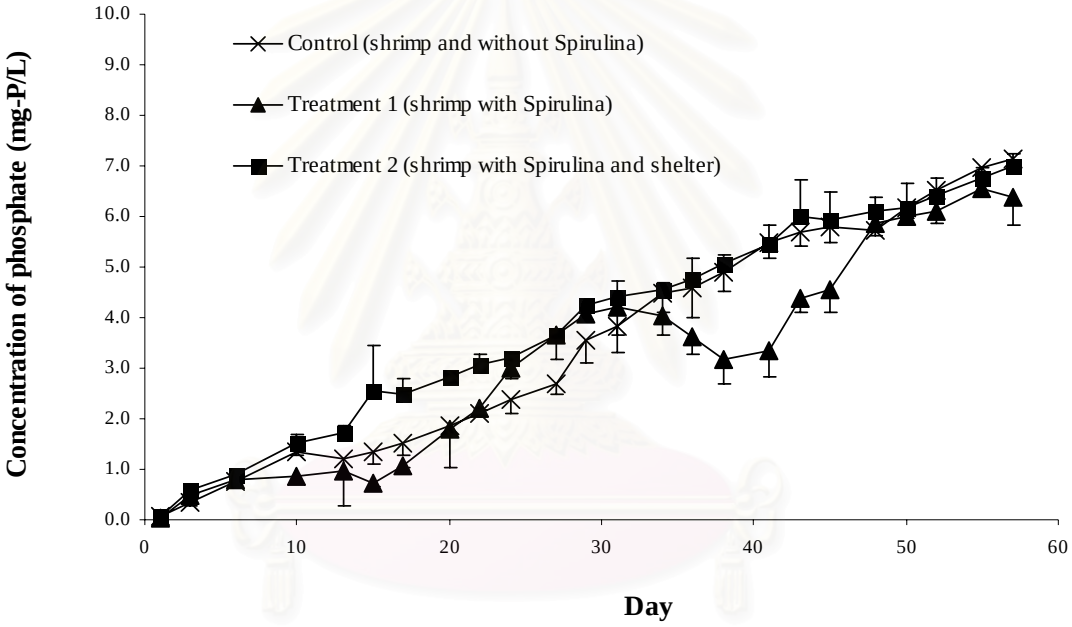

Figure 5.5 Average phosphate concentrations during the experiment in control (shrimp and without *S. platensis*), treatment 1 (shrimp with *S. platensis*), and treatment 2 (shrimp with *S. platensis* and shelter) during the experiment.

During experimental period, pH was between from 8.5-9.1 and maximum temperature in the afternoon was between 25-29°C. Average salinity was 30 psu. Average dissolved oxygen was 5.45 ± 0.32 mg L^{-1} (**Table 5.2**). Average alkalinity was 160 (120-230 mg $CaCO_3$ L^{-1}, **Fig. 5.6**). There was no significant different (P>0.05) between water quality of control and all treatments. All mentioned parameters were within the acceptable range for growth of shrimp (Fast, 1992a, b; Chanratchakool *et al*., 1998) and *Spirulina* (Richmond, 1986; Vonshak, 1997).

Table 5.2 Chemical parameter of water quality in the experiment.

Parameters	Experiment		
	Control	Treatment 1	Treatment 2
pH	8.54-8.91	8.74-9.12	8.54-8.91
Temperature (°C)	26.80-28.95	25.30-28.05	26.80-28.95
Salinity (psu)	28-30	28-30	28-30
Alkalinity (mg as $CaCO_3$ L^{-1})	139.5-223.0	127.0-223.0	138.5-225
Dissolved oxygen (mg L^{-1})	5.14-5.91	5.43-6.24	5.78-6.58

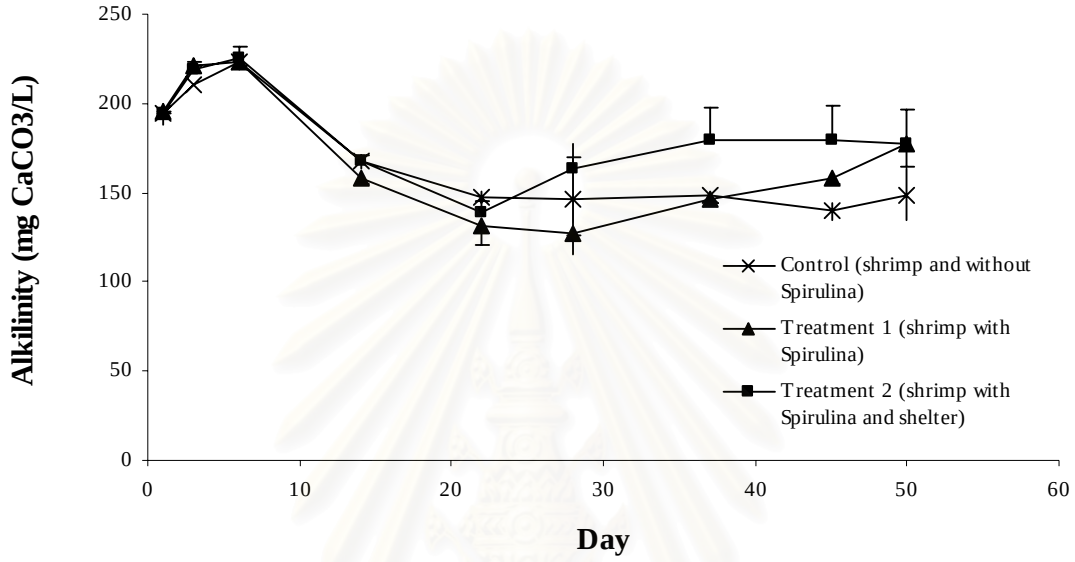

Figure 5.6 Average alkalinity of control (shrimp and without *S. platensis*), treatment 1 (shrimp with *S. platensis*), and treatment 2 (shrimp with *S. platensis* and shelter).

Concentration of chlorophyll-a (Chl-*a*) which indicated the number of phytoplankton in each tank is shown in **Fig. 5.7**. For control, Chl-*a* was between 0.015 and 0.05 mg L^{-1}. This indicated the fluctuation of natural phytoplankton population found in the tank. On the other hand, number of *S. platensis* trichome in treatment 1 and 2 as showed in **Fig. 5.8** was not related with Chl-*a* especially during the first 30 days. Cloudy weather during day 1-20 possibly one of the factor that limited growth of *S. platensis*.

After day 25, *S. platensis* became a dominant phytoplankton species in treatment 1 and semi-continuous harvesting was done in day 36, 37, 38, 41, 52, 55, 56, and 57. Unfortunately, number of *S. platensis* in treatment 2 (shrimp + *S. platensis* + shelter) was much lower than in treatment 1. This might due to nutrient limitation since TIN in treatment 2 was less than half of that in treatment 1 **(Fig. 5.9, and 5.10;** for control showing in **Fig. 5.11)** plus limitation of light shading under the shelter.

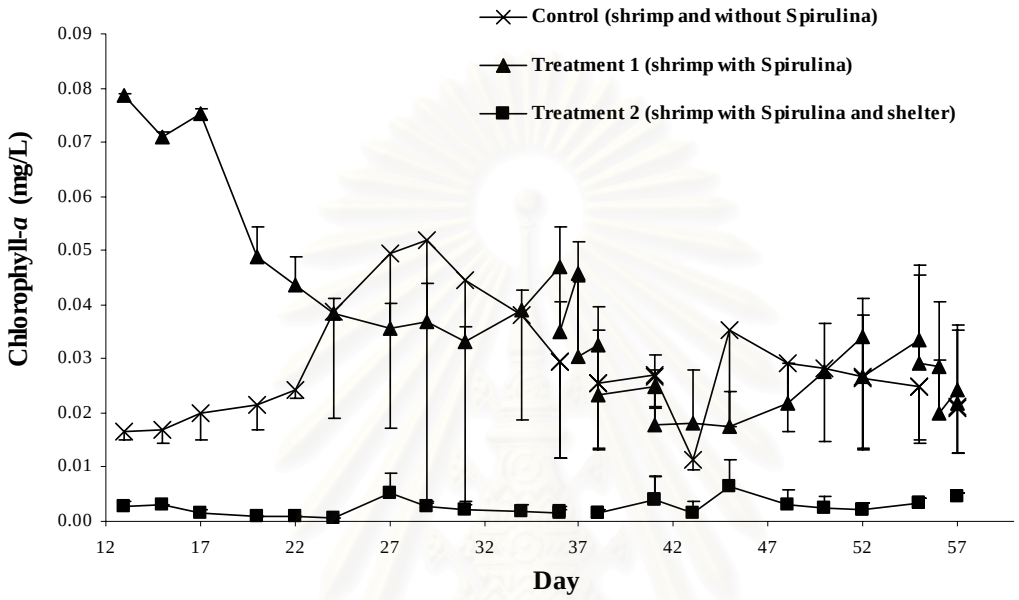

Figure 5.7 Chlorophyll-*a* concentrations in shrimp culture tanks; control (shrimp and without *S. platensis*), treatment 1 (shrimp with *S. platensis*), and treatment 2 (shrimp with *S. platensis* and shelter) during the experiment. Error bars indicate standard deviation with *n*=2. For treatment 1, error bars were from chlorophyll-*a* data before harvesting.

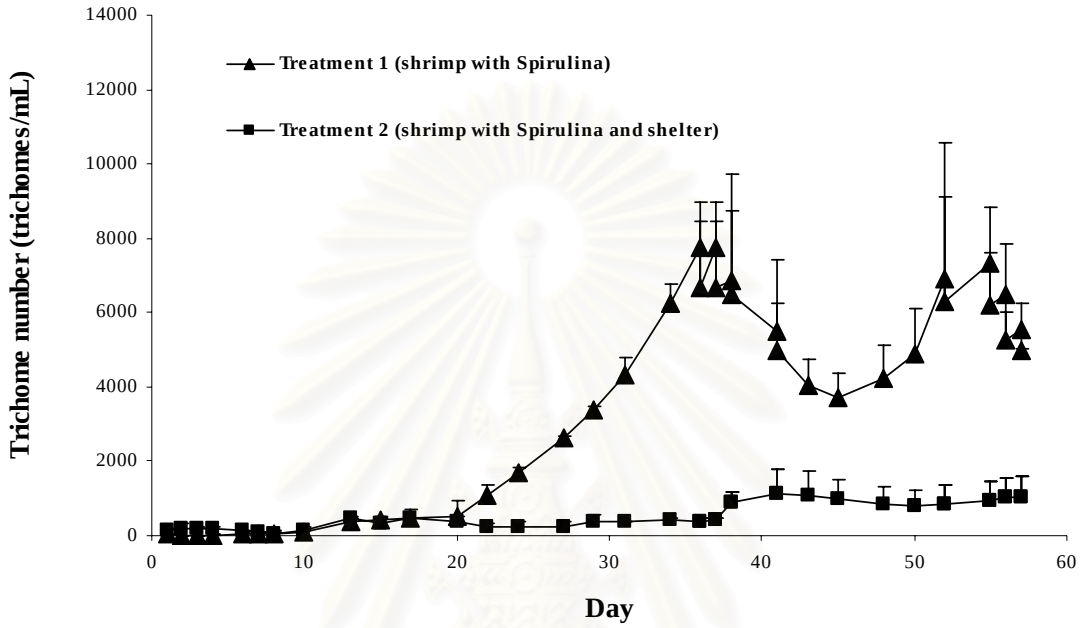

Figure 5.8 Trichomes number in shrimp culture tanks; treatment 1 (shrimp with *S. platensis*), and treatment 2 (shrimp with *S. platensis* and shelter) during the experiment. Error bars indicate standard deviation with *n*=2. For treatment 1, error bars were from trichome data before harvesting.

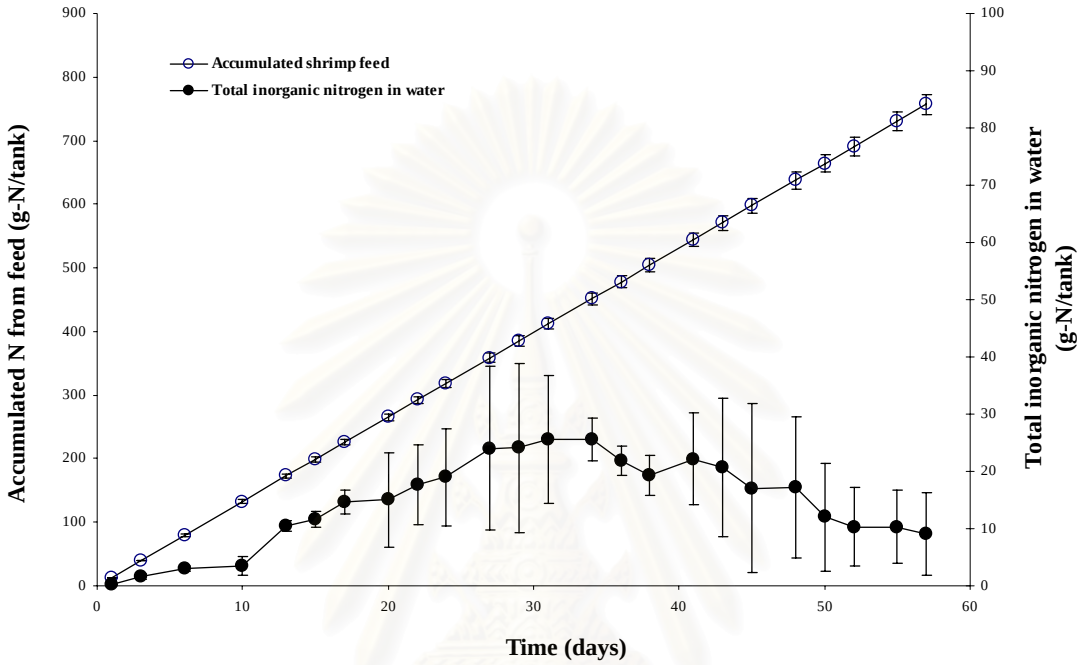

Figure 5.9 Relationship between estimated nitrogen accumulation from feed and total inorganic nitrogen concentration in water of treatment 1 (shrimp with *Spirulina*).

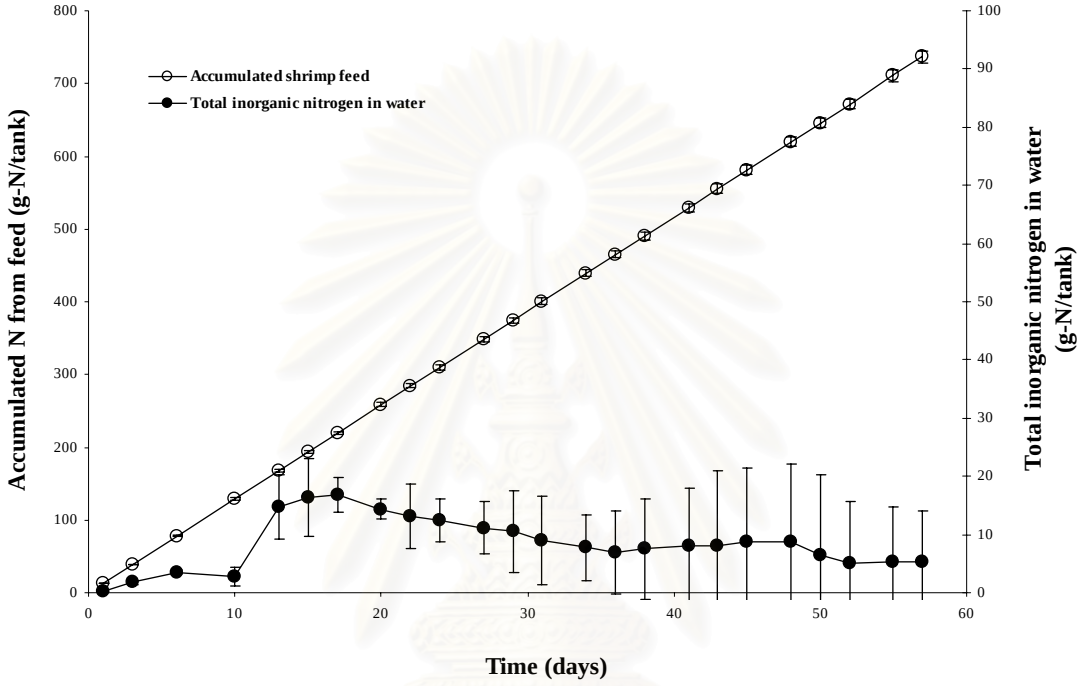

Figure 5.10 Relationship between estimated nitrogen accumulation from feed and total inorganic nitrogen concentration in water of treatment 2 (shrimp with *Spirulina* and shelter).

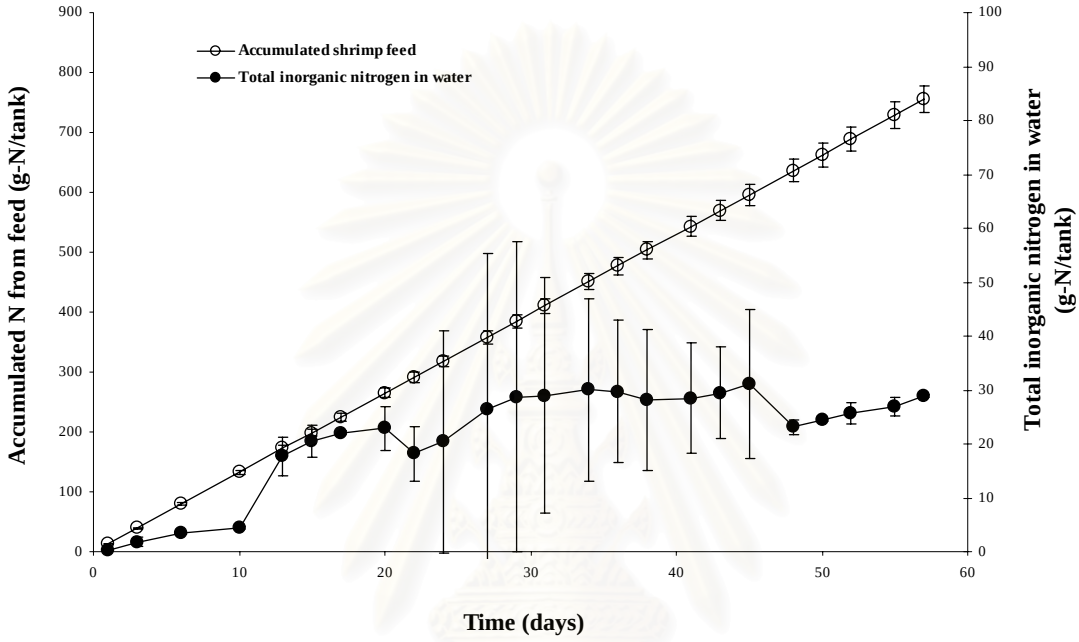

Figure 5.11 Relationship between estimated nitrogen accumulation from feed and total inorganic nitrogen concentration in water of control (shrimp without *Spirulina*).

Phytoplankton assimilation is generally considered as the most important process that eliminates nitrogen from earthen ponds (Burford and Glibert, 1999). Moreover, Epp (2002) reported that when NH_4Cl was added directly to the tanks, the algae rapidly incorporated NH_4^+ into cellular material. According to Hargreaves (1998), estimated nitrogen uptake by phytoplankton ranged from 150 to 450 mg N m^{-2} day^{-1} in temperate aquaculture ponds.

In general, nitrogen from feed was incorporate into shrimp biomass and some was excreted as ammonia. Uneaten feed and feces were decomposed in sediment bottom which later converted into ammonia or ammonium. To prevent or reduce ammonia accumulation in the culture system, ammonia was usually transformed into nitrite and nitrate through nitrification process. Loss of ammonia from the culture system could also happen in other ways such as ammonia assimilation in plant or by volatilization directly to the atmosphere.

In **Figure 5.12**, postulated pathway of nitrogen cycle in this study indicated that harvesting of plant is the most feasible process while volatilization and denitrification were not easy to manage. However, nitrogen elimination through settle solid harvesting could be an alternative choice. Using the data in treatment 1, after combination nitrogen in *Spirulina* (4.88%), other sessile algae (8.25%) and settle solid (10.89%), up to 24% of nitrogen could possibly be removed during culture period (see 5.3.3.2 Table 5.5).

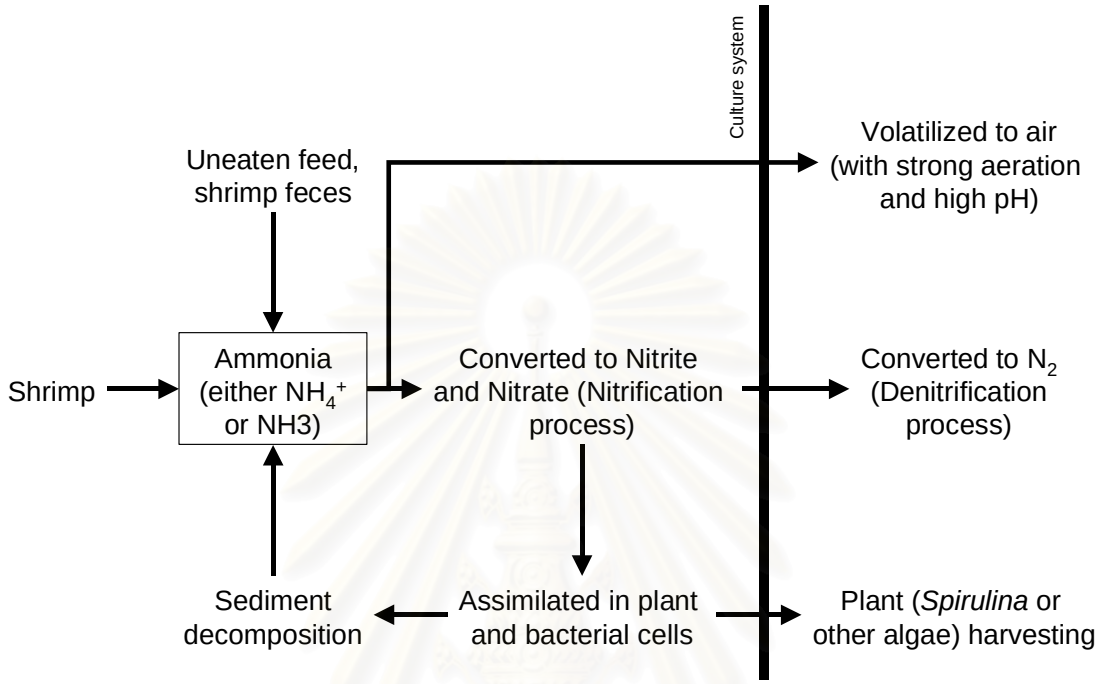

Figure 5.12. Postulated pathway of nitrogen cycle in this study.

The major source of nitrogen compounds in aquaculture pond were direct excretion of shrimp and decomposition of organic matter and uneaten shrimp feed (Avult, 1993). Kou and Chen (1991) mentioned that ammonia and nitrite in the intensive shrimp culture systems increased exponentially overtime. Chen and Tu (1991) reported that ammonia might increased to 6.5 mg NH_3-N L^{-1} even with frequent water exchange in *Penaeus monodon* grow out system. In this study, however, ammonium of all treatments including control was never exceed the acceptable concentration for aquaculture (Boyd, 1990 and Chen and Tu, 1991).

Two possible processes that reduced TIN of treatment 2 were, firstly, nitrogen uptake by other sessile macroalgae naturally found attach to the shelter and, secondly, denitrification process that occurred in microbial biofilm at the shelter surface and in the settled sediment. In fact, nitrogen content in sessile algae and sediment could be determined at the end of the experiment, however, nitrogen loss by denitrification was include in unidentified source.

Krom and Neori (1989) mentioned that nutrients, together with the high light intensity and warm temperature, support active growth of phytoplankton. Growth of *Spirulina* in treatment 1 (without shelter) was detected during the experiment but only in short period. In treatment 2 (with shelter), chlorophyll-*a* and trichome number of *Spirulina* were substantially lower than in treatment 1 and even lower than in control. Since at least 45% of tank surface area was shaded by the shelter. When combine the shading effect with high turbidity water in the tanks, sunlight could penetrate only a few centimetre below the water surface. This could be the most important limiting factor to *Spirulina* photosynthesis and growth. As recorted in the fish pond, gross productivity rate was found highest near the water surface and declined rapidly with depth because dense phytoplankton standing crop reduced light penetration (Lin, 1986). On the other hand, this condition unlikely promoted growth of sessile macroalgae that grew attach in to the shelter top and tank surface (**Fig. 5.13**). Therefore, to improve the efficiency of using *Spirulina* for water quality control, clear water system is one of the most important factors that need to be maintained. This can be achieved by proper aeration or alternative mixing systems that allowed some sedimentation at the bottom of the tank.

Figure 5.13 Contaminated sessile macroalgae found in this experiment.

In this experiment, nitrite concentration was very high in all treatments. The highest concentration up to 30 mg NO_2-N L^{-1} was detected in control tanks. These concentrations were extremely high and could affect shrimp physiology. In normal condition, nitrite is never found accumulate in high dissolved oxygen environment since it will immediately be converted into nitrate. Accumulation of nitrite in this experiment could possibly relate with two processes, incomplete nitrification or incomplete denitrification. The pathway of nitrite accumulation found in this experiment is could be summarised as in **Fig. 5.14.** It need to be stated that nitrite concentration found in this experiment was exceptionally high. Toxicity of nitrite as reviewed by Boyd (1990) could affect shrimp around 8.5 – 15.4 mg NO_2-N L^{-1}, therefore, high nitrite concentration was one of the factor that induce stress and affect shrimp growth and survival. As this experiment was started using extreamly high density (120 shrimp m^{-2} bottom area) of moderate size shrimp (4.5 g), this density could not be compared with normal shrimp culture which range from 25 to 50 shrimp m^{-2} at post-larvae 15, (0.7 g shrimp).

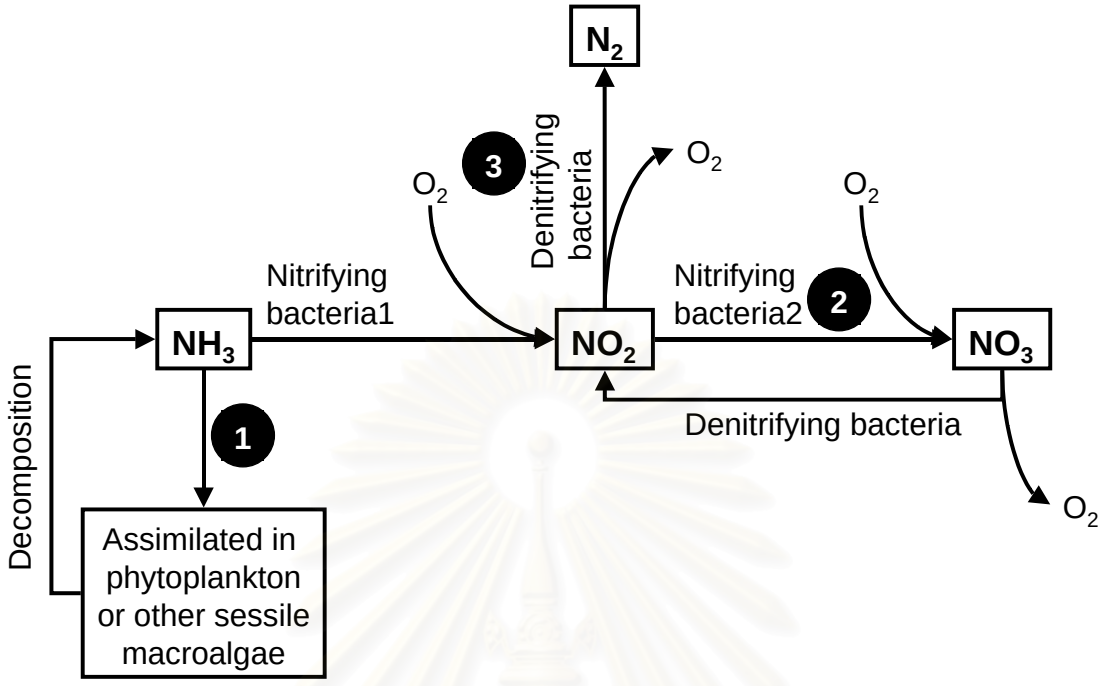

Figure 5.14 Pathway of nitrite accumulation possibly occurred in this experiment. [1] Ammonia (NH_3) was partly assimilated in the algae and most of ammonia was converted to nitrite (NO_2^-). [2] Incomplete nitrification possibly caused nitrite accumulation. [3] Incomplete denitrification process also increased nitrite concentration in the water.

In theory, incomplete nitrification happen when nitrite can not be converted to nitrate. This cause by factors that limiting activity of nitrite reducing bacteria *i.e. Nitrobactor*, *Nitrospira* and others. Two important factors are low oxygen concentration and low alkalinity (Bitton, 1994; Zweig *et al.*, 1999; Ruiz *et al*., 2003). As nitrification is carried out in two steps, Ruiz *et al*. (2003) suggested that 75% of total oxygen requirement is needed for ammonia oxidizing bacteria in the first step (equation 4). Another 25% of oxygen requirement is then needed to complete the second step by

means of nitrite oxidizing bacteria (equation 5). However, Ruiz *et al.* (2003) found that DO range from 5.7 to 1.7 mg L^{-1} did not affected nitrification process in their experiment while nitrite accumulation took place when setting DO concentration at 0.7 mg L^{-1}. This low DO is possibly happen in the around the surface of the tank and sheter.

$$NH_4^+ + {}^3/_2O_2 \rightarrow NO_2^- + H_2O + 2H^+$$

(Ammonia oxidizing bacteria require 1.5 mol of oxygen per mol of nitrogen)....(4)

$$NO_2^- + {}^1/_2O_2 \rightarrow NO_3^-$$

(Nitrite oxidizing bacteria require 0.5 mol of oxygen per mol of nitrogen).....(5)

Incomplete denitrification (see number 3 in Fig.5.16) on the other hand, is usually found when oxygen concentration in outer layer of biofilm was reduced to 1-2 mg L^{-1} by bacterial respiration (Ruiz *et al*., 2003). This oxygen concentration might just proper for the first reducing step of denitrification process but was not low enough to complete the process since there was continuous aeration in the tank. According to Van Rijn (1996), nitrite accumulatation could be found in the recirculating water system containing high nitrate concentration. In intensive fish culture systems, nitrite was accumulated as a result of incomplete denitrification at low oxygen concentrations, or where denitrification was inhibited by limitation of organic matter (van Rijn and Rivera, 1990; van Rijn and Sich, 1992). Moreover, lack of carbon source for denitrifying bacteria might also another factor that affect denitrification process. Results from

denitrification studies published elsewhere such as Christensen and Harremoes (1978), Spotte (1979), Yang *et al.* (1989), Whitson *et al*. (1993), Bitton (1994), Aboutboul *et al*., 1995, Gross *et al.* (2000) and Menasveta *et al*. (2001), confirmed that carbon addition is essential for complete denitrification process in aquaculture system. Since there was not enough data to complete the explanation of nitrite accumulation found in this study, it has to be stated that nitrite could possibly come from either incomplete nitrification or incomplete denitrification processes.

Nitrate concentrations in both algae contained tanks (treatment 1 and 2) were lower than in control. Hence, it could be stated that ammonia was partly assimilated in the algal cells. Apart from algal assimilation, dissolved ammonia was then converted into nitrite by nitrification process under aerobic condition. On the other hand, the condition of this experiment was also a combination of both aerobic and anaerobic environment. In the water body, oxygen concentration was high due to continuous aeration but anaerobic environment could possibly happen in the biofilm layer on the tank surface and also in the sediment (including settle sediment). In treatment 1 (shrimp with *S. platensis*), surface area for bacterial biofilm was lower than in treatment 2 (shrimp with *S. platensis* and shelter). Therefore, complete denitrification process could possibly occur in biofilm layer of the shelter and this might be one of the factor that eliminated nitrogen out of the system.

5.3.2 Shrimp growth, survival and production yield

Summary of shrimp weight gain and food conversion is showed in **Table 5.3**. At the end of experiment, average shrimp weight in control, treatment 1 and treatment 2 were 5.63 ± 0.33, 6.45 ± 0.23 and 7.04 ± 0.04 g, respectively. Statistical analysis using ANOVA (**Fig. 5.15**) indicated that average weight of shrimp on days 45 and day 57 of the two treatments groups were not significantly different (P>0.05), but both treatments had significantly greater weight than the control group on day 57 (P<0.05).

Table 5.3 Summary of shrimp weight gain, survival, biomass production, total feeding and food conversion (FCR) in this experiment.

Parameters	**Control (no *Spirulina*, no shelter)**	**Treatment 1 (with *Spirulina*)**	**Treatment 2 (with *Spirulina* and shelter)**
Average Daily Growth (ADG g day^{-1})	0.019 ± 0.014	0.034 ± 0.019	0.047 ± 0.025
Survival Rate (%SR)	30.56 ± 1.96	46.53 ± 0.98	67.37 ± 0.98
Final Mean Body Weight (^{Final}MBW g)	5.63 ± 0.33	6.45 ± 0.23	7.04 ± 0.04
Biomass Produced (g)	124.05 ± 15.13	216.08 ± 12.27	341.55 ± 6.72
Total feed used (g)	755.30 ± 22.93	757.13 ± 15.33	736.46 ± 7.93
Food Conversion Ratio (FCR)	6.15 ± 0.93	3.51 ± 0.27	2.16 ± 0.07

Values are mean ± S.D. (n=2)

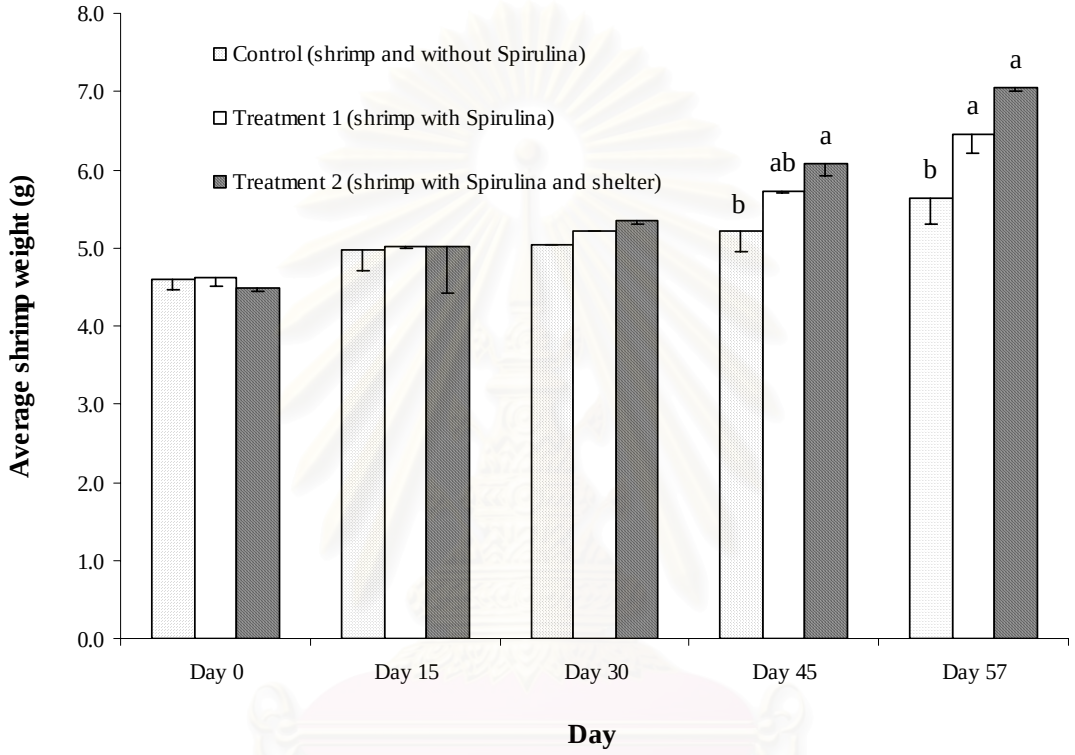

Figure 5.15 Average shrimp weights of control (shrimp without *S. platensis*), treatment 1 (shrimp with *S. platensis*) and treatment 2 (shrimp with *S. platensis* and shelter). Error bars represent standard deviation (n=2) while a or b indicate significant difference (P<0.05).

On the other hand, average shrimp survival at the end of experiment was only 30.6%, 46.5% and 67.4% for control, treatment 1 and treatment 2, respectively. Treatment 2 had the highest survival rate and significantly different ($P<0.05$). Treatment 1 had significantly ($P<0.05$) higher survival rate than control group.

It was found that the highest survival rate was in shrimp that cultured with *S. platensis* and shelter (treatment 2). This indicated the advantage of using shelter to reduce carnivorous behavior of shrimp and increase surface area for shrimps attachment. However, survival rate of high density shrimp culture without shelter (treatment 1) was still higher than in control. This suggested that the co-culture of *Spirulina* with shrimp could better maintain shrimp survival even at very high culture density. On the other hand, survival rate of control (without *Spirulina*) was significantly lower than other two treatments. For treatment 1 and 2, activity of algae including *Spirulina* and other sessile algae could maintain better water quality that affect shrimp survival. The average FCR content of treatment 2 in this study was 2.16, closely with other species culture in cement tank such as juvenile *Penaeus vannavei* (2.0-2.5 according to Wyban and Sweeney, 1989). Likewise, Funge-Smith and Briggs (1998) stated that FCR of shrimp culture in Earthern pond in Thailand ranged from 1.8 to over 2.0.

In the previous chapter, co-culture of shrimp with *Spirulina* had showed the excellent performance in water quality control in an experiment using clear water aquarium. The condition in this experiment was in the different manner since very high density of shrimp (120 shrimp m^{-2}) was used. This concentration was much higher than

the traditional intensive culture of shrimp in Thailand that usually has around 50-100 shrimp m^{-2} (Briggs and Funge-Smith, 1994) but less than 50 shrimp m^{-2} was the most exceptional density (Allan and Maguire, 1992). These densities were still high in comparison with typical shrimp culture. With this high density, shelter in treatment 2 was then provided to increase attaching area for shrimp. Observation during the experiment confirmed that most of shrimps were likely to stay within the shelter rather than swimming around the bottom of the tank. This probably made treatment 2 the highest shrimp survival rate.

5.3.3 Nitrogen and phosphorus budget

5.3.3.1 Proximate analysis of nitrogen and phosphorus in the culture system

Proximate analysis of nitrogen and phosphorus content in dry matters from the last day is showed in **Table 5.4** These table were therefore used to calculate total nitrogen and phosphorus in each tank at the beginning and at the end of the experiment. Moreover, sum of dissolved inorganic nitrogen *i.e.*, ammonium, nitrite and nitrate-nitrogen was used to indicate total dissolved nitrogen.

Table 5.4 Quantitative analysis of nitrogen and phosphorus in dry matters found in the culture system

Source	% Protein	% Nitrogen	% Phosphorus
Shrimp feed	36.56 ± 1.71	5.85 ± 0.27	0.81 ± 0.01
Shrimp carcass	69.18 ± 1.49	11.07 ± 0.24	1.16 ± 0.17
S. platensis biomass	56.44 ± 3.46	9.03 ± 0.55	1.83 ± 0.98
Other algae (sessile form)	24.29 ± 1.01	3.89 ± 0.16	0.44 ± 0.05
Shrimp molting	33.46 ± 1.37	5.35 ± 1.37	0.31 ± 0.01
Settle solid	27.27 ± 2.98	4.36 ± 0.48	0.59 ± 0.09

The average nitrogen content of dry shrimp (*Penaeus monodon*) in this study was 11%, closely with other studies such as in *Penaeus vannavei* (11.2% according to Teichert-Coddington *et al*., 2000) and black tiger shrimp (11.5% according to Funge-Smith and Briggs, 1998). The average phosphorus content of shrimp carcass (*Penaeus monodon*) in this study was 1.16%, also closely with previous study (1.19% according to Funge-Smith and Briggs, 1998). The average phosphorus content of dry shrimp feed in this study was 0.81%, closely with 0.29-0.90% as reported in Qifeng, 1991.

5.3.3.2 Nitrogen budget

Nitrogen budget study in **Table 5.5** revealed that the major input of nitrogen was from shrimp feed and initial shrimp biomass. Nitrogen input through shrimp feed, ranged from 51 to 53% of total input. These figures were lower than among the normal range as

previous published reports such as 82% by Muthuwan (1991) an 94% by Satapornvanit (1993). The rest of input was from shrimp biomass which accounted from 17 to 48% of total input. Nitrogen in the water at the beginning was very low in all treatments (0.18 to 0.25%). In addition, nitrogen input as *Spirulina* biomass in treatment 1 and 2 was only 2.01 and 3.52%, respectively.

As shown in Table 5.5, the major portion of the nitrogen in control tanks was deposited as dissolved inorganic nitrogen. On the other hand, nitrogen of both treatments was deposited in shrimp, sediment and algae while relatively smaller fraction were retained by molting. Nitrogen assimilated in *Spirulina* of treatment 1 was accounted by 4.88% of total nitrogen output, less than that absorbed by other sessile macroalgae (8.25%). These sessile macroalgae occurred naturally around the edge of the tanks during the experiment. In treatment 2, sessile macroalgae was found not only at the tank wall but also attach to the shelter surface. As the result, the largest portion of 47.64% nitrogen output was therefore absorbed by shrimp following by sessile macroalgae (14.44%), sediment (6.81%) and water (6.30%) respectively.

Table 5.5 Nitrogen budget for different treatments during 57-day experiment

Source	Control (no *Spirulina*, no shelter)	Treatment 1 (with *Spirulina*)	Treatment 2 (with *Spirulina* & shelter)
Nitrogen Input			
Shrimp (g-N per tank)	36.67 ± 1.11	36.76 ± 0.74	35.76 ± 0.39
(%)	***(46.36 ± 2.64)***	***(45.45 ± 2.15)***	***(44.74 ± 1.12)***
Shrimp feed (g-N per tank)	42.24 ± 1.28	42.34 ± 0.86	41.19 ± 0.45
(%)	***(53.40 ± 0.02)***	***(52.36 ± 0.22)***	***(51.53 ± 0.15)***
Spirulina (g-N per tank)	0	1.62 ± 0.28	2.81 ± 0.26
(%)	***(0)***	***(2.01 ± 0.38)***	***(3.52 ± 0.27)***
Water (g-N per tank)	0.19 ± 0.03	0.14 ± 0.03	0.17 ± 0.01
(%)	***(0.25 ± 0.05)***	***(0.18 ± 0.04)***	***(0.21 ± 0.01)***
Nitrogen Output			
Shrimp (g-N per tank)	13.73 ± 1.68	23.92 ± 1.36	38.07 ± 0.37
(%)	***(17.40 ± 2.64)***	***(29.59 ± 2.15)***	***(47.64 ± 1.12)***
Spirulina (g-N per tank)	0	3.96 ± 1.61	0
(%)	***(0)***	***(4.88 ± 1.91)***	***(0)***
Settle solid (g-N per tank)	8.69 ± 0.40	8.82 ± 0.96	5.44 ± 0.16
(%)	***(10.98 ± 0.18)***	***(10.89 ± 1.01)***	***(6.81 ± 0.29)***
Water (g-N per tank)	30.03 ± 0.41	7.96 ± 3.24	5.06 ± 3.04
(%)	***(37.97 ± 0.61)***	***(9.88 ± 4.16)***	***(6.30 ± 3.72)***
Other algae (g-N per tank)	0	6.68 ± 0.35	11.52 ± 1.92
(%)	***(0)***	***(8.25 ± 0.30)***	***(14.44 ± 2.60)***
Molting (g-N per tank)	0.04 ± 0.02	0.13 ± 0.01	0.20 ± 0.01
(%)	***(0.05 ± 0.03)***	***(0.16 ± 0.01)***	***(0.25 ± 0.01)***
Unidentified (g-N per tank)	26.62 ± 3.20	29.41 ± 2.98	19.63 ± 0.52
(%)	***(33.61 ± 3.04)***	***(36.35 ± 3.10)***	***(24.56 ± 0.31)***

Values are mean ± S.D. (n=2)

Briggs and Funge-Smith (1994) suggested that, although high protein content feed was used, only 20-23% of nitrogen from feed was then retained in shrimp biomass. Most of nitrogen (80%) was then excreted to surrounding environment. At the end of typical shrimp culture, 27-57% of total nitrogen was discharged from the pond by water exchange (Funge-Smith and Briggs, 1998; Burford and Williams (2001). According to Teichert-Coddington *et al*. (2000), at least 59% of nitrogen from feed was finally waste the pond. Martin *et al*. (1998) also mentioned that feeding in shrimp pond resulted in nitrogen accumulation in the sediment, so called the nitrogen sink (Zur, 1981; Alongi *et al*., 2000; Jamu and Piedrahita, 2002).

Muthuwan (1991) stated that the major output of nutrients in shrimp ponds was released in discharged water. Likewise, total nitrogen in drained water of this study during harvesting were 37.97, 9.88 and 6.30 % nitrogen for control, treatment 1 and treatment 2, respectively. Teichert-Coddington *et al.* (2000) reported that 80% of total nitrogen loss from the pond occurred primarily from daily water exchange (72%) and pond drainage (8%). Boyd (1995) suggested that traditional pond management practices often included fertilization to promoted primary productivity. This practice is therefore confusing since most of primary nutrients for phytoplankton in the pond are readily supplied by fish excretion and feed decomposition. It was at least to be mentioned that algae in both benthic and planktonic forms could successfully reduce nitrogen waste in the discharged water from the culture system and the major sink of nitrogen in this study were sessile macroalgae (14%) in treatment 2 and suspended solid (8.25%) in treatment 1 and control.

Unidentified nitrogen was among the largest portion of nitrogen after balancing nitrogen output with the input. In this experiment, unidentified nitrogen ranged from 33% in control to 36% in both treatments. This unidentified or unaccounted source was generally found between 9 to 50% of nutrient budget in many reports elsewhere (Funge-Smith and Brigges, 1988; Lefebvre *et al*., 2001; and Thoman *et al*., 2001). Daniels and Boyd (1989) estimated that >50% of the nitrogen input via feed could be lost through the combined effects of denitrification and ammonia volatilization in polyethylene-lined, brackish water ponds. In this experiment, evaluation of denitrification process was not included in the methodology since there was not soil bottom layer. However, accumulation of nitrite found during experiment indicated a possibility of denitrification in biofilm layer of both tank wall and shelter surface. According to Thoman *et al.* (2001), removal of nitrogen through denitrification was the most likely explanation for 9-21% of nitrogen loss from closed recirculating mariculture systems. According to Gross *et al.* (2000), denitrification rate varies with temperature, pH, abundance of denitrifying bacteria, concentration of nitrate, organic carbon, and dissolved oxygen. In addition, Gross *et al.* (2000) illustrated that denitrification in aquaculture pond was approximately 38 mg N m^{-2} d^{-1}. In typical earth ponds, denitrification ranged from 17.4-57.3% of nitrogen output were recorded (Gross *et al.*, 2000). Denitrification up to 100 kg hr^{-1} yr^{-1} was reported by Olah and Pekar (1995) in an integrated fish culture pond. Shrimp culture in concrete tank without soil bottom at 50 shrimp m^{-2} conducted by Thakur and Lin (2003) also found that there was 36% of unaccounted nitrogen after balancing nutrient butget. Another treatment of the same report, shrimp culture in concrete tank with sediment bottom, had much lower

unaccounted nitrogen (only 5.2%). This strongly suggested that bottom sediment is one of the important factor for nitrogen removal process. Ammonia volatilization, as mentioned in Daniels and Boyd (1988) and Thakur and Lin (2003), might enhanced the lost of nitrogen in the tank with vigorous aeration and high pH. Schroeder (1987) indicated that ammonia volatilization rate could be up to 50 mg N m^{-2} day^{-1},depending on the concentration of unionized ammonia, temperature, salinity as well as evaporation rate and wind speed (Hargreaves, 1998). Moreover, since dissolved organic nitrogen (DON) analysis was not included in this experiment because of technical problem, DON might be another possible source of unidentified nitrogen in the culture system.

Nitrogen budget in this study showed that 17-48% of nitrogen was incorporated into harvested shrimp, closely with other publications such as 22.8-30.7% in Thakur and Lin (2003); 22% in Brigges and Funge-Smith (1994), or even 20 to 26% of nitrogen output from fish pond (Daniels and Boyd, 1989; Krom and Neori, 1989). In this study, statistical analysis revealed that there was no significant difference (P<0.05) in total feed input and also in total nitrogen input which ranged from 79.1 to 80.86 g-N per tank. Low shrimp biomass therefore related with slow growth rate and low survival rate of shrimps in all treatments as possibly the effect of high nitrite concentration as previously discussed. This was mainly the result of low survival rate in all treatments.

5.3.3.3 Phosphorus budget

As shown in **Table. 5.6**, phosphorus budget in all treatments related with nitrogen budget. The major input of phosphorus was also from shrimp feed (56-60%) and initial shrimp biomass (37-39%). Phosphorus input from *Spirulina* was 3.27% and 5.66% in treatment 1 and treatment 2, respectively. Phosphorus input from water, however, was very low with only 0.2-0.3% of total input.

In the present study, average phosphorus content in *Penaeus monodon* was 1.16 %, close to phosphorus in *Penaeus vannamei* that was 1.25 %. (Teichert-Coddington *et al*., 2000). Feed contributed 56 to 60% of total phosphorus input (see Table 5.5) which was lower than the previous reports such as 87% in Muthuwan (1991) or 91% in Satapornvanit (1993). Phosphorus content in shrimp biomass of this study ranged from 14-39% higher than 4.9-7.7 % in Brigges and Smith (1994) and 9% in Teichert-Coddington *et al*. (2000).

Table 5.6 Phosphorus budget for different treatments during 57-day experiment

Source	Control (no *Spirulina*, no shelter)	Treatment 1 (with *Spirulina*)	Treatment 2 (with *Spirulina* & shelter)
Phosphorus Input			
Shrimp (g-P per tank)	3.84 ± 0.12	3.85 ± 0.08	3.75 ± 0.04
(%)	***(39.42 ± 0.02)***	***(38.15 ± 0.25)***	***(37.18 ± 0.15)***
Shrimp feed (g-P per tank)	5.88 ± 0.18	5.89 ± 0.12	5.73 ± 0.06
(%)	***(60.30 ± 0.03)***	***(58.35 ± 0.38)***	***(56.86 ± 0.23)***
Spirulina (g-P per tank)	0	0.33 ± 0.06	0.57 ± 0.05
(%)	***(0)***	***(3.27 ± 0.61)***	***(5.66 ± 0.44)***
Water (g-P per tank)	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.01
(%)	***(0.28 ± 0.05)***	***(0.23 ± 0.02)***	***(0.31 ± 0.06)***
Phosphorus Output			
Shrimp (g-P per tank)	1.44 ± 0.18	2.51 ± 0.14	3.96 ± 0.08
(%)	***(14.80 ± 2.24)***	***(24.84 ± 1.75)***	***(39.32 ± 1.36)***
Spirulina (g-P per tank)	0	0.84 ± 0.34	0
(%)	***(0)***	***(8.27 ± 3.26)***	***(0)***
Settle solid (g-P per tank)	1.31 ± 0.02	1.19 ± 0.04	0.65 ± 0.02
(%)	***(13.44 ± 0.16)***	***(11.81 ± 0.28)***	***(6.46 ± 0.27)***
Water (g-P per tank)	3.42 ± 0.12	3.06 ± 0.27	3.36 ± 0.12
(%)	***(35.13 ± 0.17)***	***(30.31 ± 3.07)***	***(33.30 ± 0.70)***
Other algae (g-P per tank)	0	0.76 ± 0.04	1.30 ± 0.22
(%)	***(0)***	***(7.48 ± 0.29)***	***(7.29 ± 2.35)***
Molting (g-P per tank)	0	0.01 ± 0.00	0.01 ± 0.00
(%)	***(0.02 ± 0.01)***	***(0.07 ± 0.01)***	***(0.11 ± 0.01)***
Unidentified (g-P per tank)	3.57 ± 0.32	1.74 ± 0.13	0.79 ± 0.34
(%)	***(36.62 ± 2.22)***	***(17.23 ± 1.01)***	***(7.86 ± 3.27)***

Values are mean ± S.D. (*n*=2)

In general, unaccounted phosphorus in the earth pond is mostly the result of mud adsorption, as mud have a strong affinity to phosphorus (Boyd, 1985). Up to 84% of phosphorus input in shrimp pond was found deposite in bottom sediment (Funge-Smith and Brigges, 1998). Qifeng (1991) reported that 34-51% of phosphorus input was accumulated at the bottom sediment of fish culture tank. Even in the tanks that did not have sediment bottom such as Thakur and Lin (2003), upto 66.7% of phosphorus was found in sediment that naturally occurred during the experiment. The result of this study found that 30-35% of phosphorus output was found in water fraction of all treatments. Algae in treatment tanks absorbed phosphorus up to 15.75% of total phosphorus in treatment 1. In detail, *Spirulina* and other sessile macroalgae in treatment 1 equally absorb phosphorus at approximately 7.48% and the rest was found in suspended solid (11%) and shrimp biomass (24%). In treatment 2, since there was low *Spirulina* biomass in both tanks, 7.29% of total phosphorus was found mainly in other sessile macroalgae. The highest number of phosphorus in shrimp biomass (39.32%) was observed in treatment 2 because it had the highest survival rate. Percentage of unidentified phosphorus output at 8-37% was lower than the percentage number that found in unidentified nitrogen output. This finding was similar to Thakur and Lin, 2003 which reported that measurable unidentified outputs were 5.2-36.0% for nitrogen, and 5.3-19.7% for phosphorus, respectively.

Muthuwan (1991) and Ritvo *et al*. (2002) stated that the main output of nutrients in shrimp ponds were in discharged water. Likewise, total phosphorus in drained water during harvest were 35.13, 30.31 and 33.30 % of phosphorus in control,

treatment 1 and treatment 2, respectively. The major sinks of phosphorus in this study were from shrimp carcass (14.80%, 24.84%, and 39.32% for control, treatment 1, and treatment 2, respectively). Another possible reason of unaccounted phosphorus might be related to practical error in settle solid collection and lost of sludge during drainage.

5.4 CONCLUSION

In the present study, efficiency of *Spirulina* for water quality control in shrimp culture tanks was evaluated using nitrogen and phosphorus budget analysis. The experiment consisted of three treatments, first, shrimp culture without *Spirulina* (control), second, shrimp culture with semi-continuous harvest of *Spirulina* (treatment 1) and, third, shrimp culture with *Spirulina* as treatment 1 in the tank with shelter (treatment 2). The results were concluded as the following:

1. In control tanks without *Spirulina*, ammonium concentration was high only during the first 10 days, thereafter, ammonium was transformed into nitrite and nitrate which were accumulated in the tank.
2. Change in ammonium concentration in both treatments resembled the control tank, however, treatment tanks with *Spirulina* had entirely lower nitrite and nitrate concentrations than in control. Moreover, nitrate concentrations in treatment 2 (shrimp with *Spirulina* and shelter) were lower than in treatment 1 (shrimp with *Spirulina* no shelter).
3. Very high nitrite concentrations were found in both control and all treatments. The highest concentrations were approximately 30, 25 and 18 mg NO_2-N L^{-1} for control, treatment 1 and treatment 2, respectively. High nitrite could possibly stress shrimp and therefore affect growth and survival rate of shrimps in this experiment.

4. Phosphate was found accumulated in the water of both control and treatments with the final concentration of 6-7 mg-P L^{-1}. However, harvest of *Spirulina* in treatment 1 showed the partial removal of phosphorus from the culture system.
5. In this experiment, shrimp in treatment 2 had the highest average daily growth, survival rate, biomass yield and food conversion efficiency, following by treatment 1 and control respectively.
6. The highest percentage of nitrogen input was come from shrimp feed (52% of total N input) following by initial shrimp biomass (45% of total N input) similarly in both control and treatments. On the other hand, the portion of nitrogen output of treatment groups was different from control. The highest nitrogen output of control was found in water (38%) while the highest nitrogen output of treatments was in shrimp (29 and 47% in treatment 1 and 2, respectively).
7. For phosphorus budget, phosphorus input source was from feed (56-60%) and shrimp (38%) in both control and treatments. Phosphorus output was found in water (35, 30 and 33%) and in shrimp (14, 24 and 39% for control, treatment 1 and treatment 2, respectively).
8. Harvest of *Spirulina* in treatment 1 removed 4.8% of nitrogen and 8.3% of phosphorus from the culture system. Unfortunately, there was no harvesting of *Spirulina* in treatment 2 due to low *Spirulina* density throughout the experiment.
9. It has to be noted that other sessile algae contaminated during the experiment had a significant portion of nutrient removal. In this experiment, up to 6-11% of nitrogen and 8% of phosphorus removal was from sessile algae.

CHAPTER VI

GENERAL DISCUSSION AND RECOMMENDATION

This study composed of three experimental chapters. The first chapter (Chapter III) involved the study on nutrient uptake kinetics of *Spirulina* in various salinity media under laboratory condition. The second chapter (Chapter IV) was the feasibility study of using *Spirulina* to control water quality in shrimp culture aquariums. The final chapter (Chapter V) was the evaluation of shrimp-*Spirulina* system in larger scale using 500L tanks with nitrogen and phosphorus budget study.

It was found that salinity had strong effect to nitrogen uptake kinetics of *Spirulina*. High salinity (30 psu) reduced more than half of ammonium and nitrate uptake rates compare to freshwater. This suggested that *Spirulina* would have lower activity when applied for nutrient treatment in seawater. The results from Chapter IV, however, indicated that *Spirulina* could effectively enough for nitrogen removal from shrimp aquarium with moderate shrimp density.

Main purpose of using *Spirulina* for water quality control in this study was to take up ammonium rather than nitrate. The nitrogen pathway released from shrimp excretion and feed decomposition must be directly assimilated in *Spirulina* as soon as possible. Therefore, the success of shrimp-*Spirulina* system is to let *Spirulina* the main nutrient remover and overcome the activity of nitrifying bacteria. This phenomenon was found during the second experiment in the aquariums (see Fig. 4.4 in Chapter IV).

However, nitrification process, as indicated by a decrease of ammonium together with an increase of nitrate concentration, naturally occurred in all experiments during the first 10-20 days of shrimp culture (Figures 4.4, 4.9 and 5.2-5.4). Increase of nitrate in those results indicated growth of nitrifying bacteria that usually took place after the incubation time of 10-20 days in the well-aerated tanks.

One of the most important questions of using *Spirulina* to take up nitrogen from shrimp culture is that what is the appropriate concentration of *Spirulina* for the system. The concentration of *Spirulina* in the system like this study must be lower than conventional *Spirulina* culture pond since too dense algae a might reduce dissolved oxygen in the pond during night and take up minerals from the water which might cause mineral deficiency in shrimp (especially for molting). For more detail, appropriate concentration of *Spirulina* should just relevant to the amount of nitrogen waste produced during shrimp culture. One of the possible way of estimating the amount of *Spirulina* need was from the balance between nitrogen input from feed and nitrogen uptake rate of *Spirulina*.

The nitrogen uptake rate in this study was derived from laboratory culture in Chapter III, although it was only a rough number, it was then calculated as the following example.

1. Estimation of nitrogen input

Assumptions:

*75% of nitrogen content in feed was excreted by shrimp (Hargraves, 1998).

Total feed used was depended on feeding program design. In Chapter IV, initial weight of shrimps was 46 g of shrimps per aquaria and shrimps were fed at 4% of total shrimp weight per day. Hence, weight of feed per day was 1.86 g. This feed contained 0.1 g of nitrogen (5.85% nitrogen content, see Table 5.4).

Nitrogen input per day (75% of nitrogen content in feed) was therefore 75% of 0.1 g nitrogen = 75 mg-N day^{-1}.

2. Estimation of appropriate *Spirulina* concentration

Assumption:

*100% of nitrogen release from shrimp culture is ammonium.

*Ammonium-nitrogen uptake rate of *Spirulina* in 30 psu salinity was assumed by half of the maximum uptake rate (½Vmax).

½Vmax of ammonium = 0.06 mg NH_4-N mgChl-*a*$^{-1}$ h^{-1} (Table 3.2)

Ammonium input = 75 mg-N day^{-1}

Spirulina concentration needed for take up ammonium = 1250 mg Chl-*a* h^{-1} or 52 mg Chl-*a* day^{-1}

Water volume in each aquaria = 30 L

Appropriate Chl-*a* concentration should be 1.7 mg Chl-*a* L^{-1}

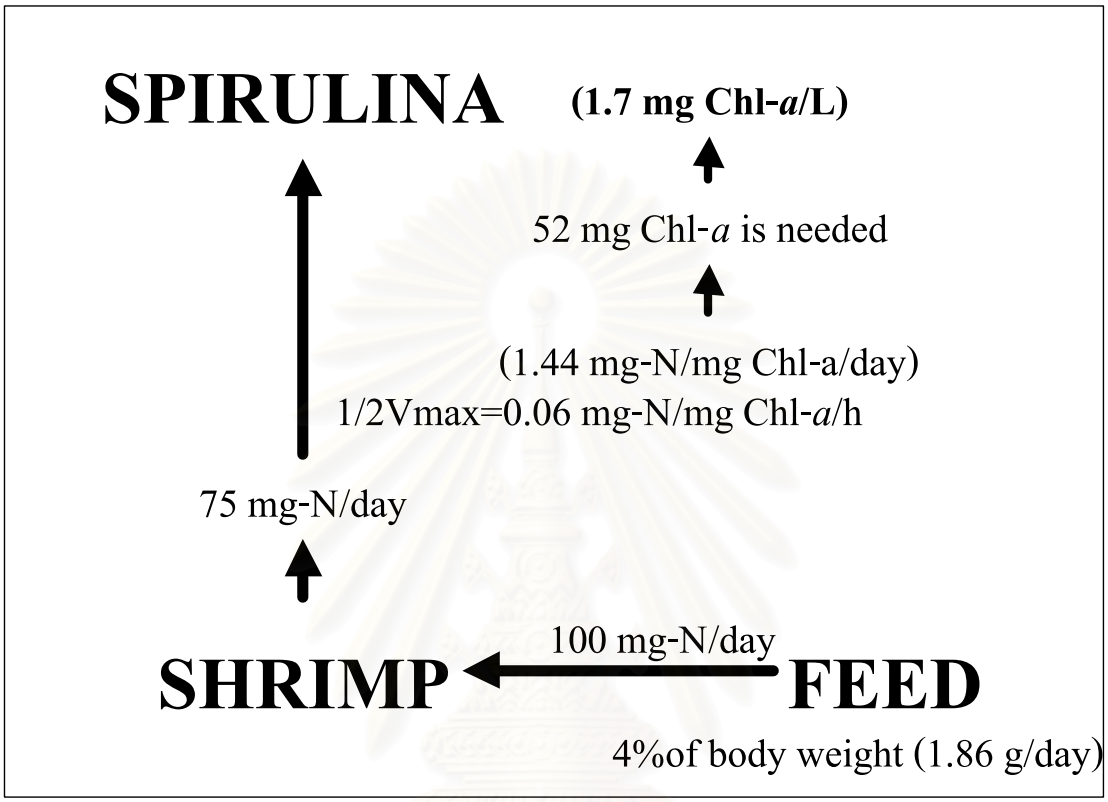

Figure 6.1 Estimation of appropriate *Spirulina* concentration for nitrogen removal from shrimp aquaria using an example data from 4.2.5 and V_{max} from Table 3.2 in trial I of this study.

Under the real experimental condition, Chl-*a* concentration in both trials in Chapter IV was maintained between 0.02-0.06 mg L^{-1} for shrimp density from 8 to15 shrimps per aquaria. This concentration was much lower than the chlorophyll requirement after the calculation above. Nitrogen excretion in the experiment in half-ton tank (Chapter V) could be estimated around 752 mg NH_4-N day^{-1}. This concentration

needed approximately 522 mg Chl-*a* $tank^{-1}$ or 1.08 mg Chl-*a* L^{-1} if the same assumption as above mentions was used. Chl-*a* concentration in the tank ranged from 0.02-0.07 mg Chl-*a* L^{-1}, as showed in Figure 5.7, was much lower than the estimated number. Hence, nitrogen removal from the system must be a combination of several processes. This indicated that there were many factors involved in ammonium removal rather than only the algal uptake. Examples of those factors are nitrification process, ammonia volatilization and nutrient assimilation in bacteria.

Although ammonium uptake rate is mainly a result of the facilitated diffusion but ammonium utilization is possibly depend on activity of the cell that is related with photosynthesis. Rate of ammonium uptake could be different between day and night. Nitrification-denitrification occurred in the culture system also involved some of ammonia and/or nitrate removal. The result of nitrification-denitrification processes was clearly found in the last experiment (in Chapter V) especially in shrimp culture with shelter.

Moreover, in Chapter V, large portion of nitrogen was assimilated in other sessile macroalgae contaminated in the tank. The use of macroalgae for nutrient removal in aquaculture was well studied (Neori *et al*. (1996, 2000); Buschmann *et al*. (1994, 1996); Olah and Peker, 1995; Troell *et al*., 1997; Nelson *et al*. (2001); Marinho-Soriano *et al*. (2002). An attempt of using *Spirulina*, as proposed in this research, differ from those macroalgae as harvesting of *Spirulina* from the pond could be done automatically using pump and 22 microns net screen. Concentration of *Spirulina* in the system could be precisely monitored using chlorophyll analysis in either extraction with solvent or

direct measurement with fluorometer. The only problem of the shrimp-*Spirulina* system is how to maintain growth of *Spirulina* in the tank. This will be a question that needed more studies in the future.

However, it must be mentioned here that nitrogen uptake rate from the study in laboratory condition was performed in an optimized medium for *Spirulina* growth. For example, Zarrouk's medium contains as high as $NaNO_3$ 2.5 g L^{-1} with complete trace elements component. Therefore, uptake rate could possibly differ from the cells found in natural water or in shrimp tank.

In addition to nutrient removal by *Spirulina,* shelter provided in treatment 2 of the experiment in Chapter V promoted high survival rate to shrimp. From observation during the experiment, shrimps were likely to stay within the shelter. This might be another technique to enhance shrimp production yield and could be used in combination with water treatment systems.

Recommendations

1. For more accurate measurement of nitrogen uptake rate, specific isotope of nitrogen might be used as an alternative method and could be performed even in the real environmental condition such as in the shrimp aquarium but the experiment need to be in special control environment.
2. Study of factors that affect growth of *Spirulina* in shrimp culture tank condition is essential for the success of shrimp-*Spirulina* system. Those factors include

physiological adaptation of *Spirulina* to various outdoor environments such as light, temperature, salinity, turbidity and nutrients.

3. Since *Spirulina* had higher nutrient uptake rate in low salinity, reducing salinity from seawater (30 psu) to brackish water (15-20 psu) might improve the activity of *Spirulina*. However, to select the proper salinity for the system, effect of salinity to shrimp physiology seems more important. For example, shrimp may have lower growth rate in low salinity but low salinity might reduce the chance of bacterial diseases that can harm to shrimp in the tank.
4. The results from this experiment indicated that sediment had high contribution to nutrient cycle in the tank. Therefore, applying shrimp-*Spirulina* system in the tank with bottom sediment still need more intense studies.
5. Ecological study of competition among phytoplankton species specially to abundance of filamentous blue-green algae in aquaculture pond would be an important information for system control and management. This includes the use of natural population of filterable microalgae rather than *Spirulina* for water quality control in aquaculture ponds.

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APPENDICES

APPENDIX A

ZARROUK MEDIUM FOR S. PLATENSIS

Growth medium for Spirulina (Zarrouk, 1966)

To 980 ml of distilled water add:

Macronutrients;

NaCl	*as needed to obtain required salinity*
$CaCl_2$	*0.04 g* L^{-1}
$NaNO_3$	*2.5 g* L^{-1}
$FeSO_4.7H_2O$	*0.01 g* L^{-1}
EDTA (Na)	*0.08 g* L^{-1}
K_2SO_4	*1.00 g* L^{-1}
$MgSO_4.7H_2O$	*0.20 g* L^{-1}
$NaHCO_3$	*16.8 g* L^{-1}
K_2HPO_4	*0.5 g* L^{-1}

And 1 ml L^{-1} *of* A_5 *and* B_6 *solution as listed below:*

Micronutrients compose of A_5 ***and*** B_6 ***solution;***

<u>A_5 solution</u>

$ZnSO_4.7H_2O$	*0.222 g* L^{-1}
$CuSO_4.5H_2O$	*0.079 g* L^{-1}
MoO_3	*0.015 g* L^{-1}

H_3BO_3	2.860 g L^{-1}
$MnCl_2.4H_2O$	1.81 g L^{-1}

B_6 solution

NH_4VO_3	229.6 x 10^{-4} g L^{-1}
$K_2Cr_2(SO_4)_4.24H_2O$	960.0 x 10^{-4} g L^{-1}
$NiSO_4.7H_2O$	478.5 x 10^{-4} g L^{-1}
$Na_2WO_4.2H_2O$	179.4 x 10^{-4} g L^{-1}
$Co(NO_3)_2.6H_2O$	439.8 x 10^{-4} g L^{-1}
$Ti_2(SO_4)_3$	400.0 x 10^{-4} g L^{-1}

Detail of preparation;

(1) Phosphorus of macronutrient should always be added last.

(2) Because of the poor solubility of NH_4VO_3, B_6 solution tends to be turbid. Thus, this solution should be well stirred before usage.

(3) Solutions A_5 and B_6 should be kept refrigerated, replacing them after 2 months.

APPENDIX B

ALL METHODS FOR PARAMETERS DETERMINATION (WATER QUALITY, TOTAL-N, TOTAL-P, CHLOROPHYLL-*a* AND CELL COUNTING)

1. Ammonia (Alternative method; Parson et al., 1989)

1.1 Outline of method

Seawater is treated in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside which acts as a catalyzer. The blue indophenol color formed with ammonia is measured spectrophotometrically at 640 nm.

1.2 Experimental procedure

1.2.1 Filter the water sample through Whatman No. 42 filter paper. Pipette 50 ml of the filtered sample into a 50 ml beaker.

1.2.2 Add 2 ml of phenol solution, swirl to mix, and when add in sequence 2 ml of nitroprusside and 5 ml of oxidizing solution; mix after each addition by swirling the flasks.

1.2.3 Allow the flasks to stand at room temperature (20-27 ˚C) for 1 hr. The top of the flask should be covered with “parafilm” during this period. The color is stable for 24 hr after the reaction period.

1.2.4 The blue indophenol color is developed in a water sample and the absorbance of the sample is measured at 640 nm. The concentration of ammonia can then be estimated by reference to calibration graph.

1.2.5 To use the blue indophenol color to estimated ammonia concentration in water samples, a calibration graph must be prepared as described in 1.2.1 to 1.2.4. The blue indophenol color is developed in solutions with know standard concentrations of ammonia and the intensity of the blue indophenol color in this series of standard sulutions is evaluated by spectrophotometry at 640 nm. Absorbance values are plotted on the Y-axis versus their respective concentrations of ammonia on the X-axis to give a calibration graph.

2. Nitrite (Parson et al., 1989)

2.1 Outline of method

The colorimetric methods generally used for nitrite employ diazotizing reagents. Nitrite reacts with reagents in acidic solution to form diazonium salts. The diazonium salts are coupled with amino or hydroxyl groups of aromatic compounds to form colored azo compound. The method employs sulfanilamide as the diazotizing reagent and N-(1-naphthyl)-ethylene-diamine as the coupling reagent. The azo compound is bright pink.

2.2 Experimental procedure

2.2.1 Filter the water sample through Whatman No. 42 filter paper. Pipette 50 ml of the filtered sample into a 50 ml beaker.

2.2.2 Add 1.0 ml of sulfanilamide solution from an automatic pipette to each 50 ml sample, mix and allow the reagent to react for more than 2 min but less than 10 min to assure a complete reaction.

2.2.3 Add 1.0 ml of naphthylethylinediamine reagent and mix immediately. Between 10 min and 2 hr afterwards, measure the extinction of the solution at a wavelength of 543 nm.

2.2.4 The concentration of nitrite can then be estimated by reference to the calibration graph.

2.2.5 To use the highly colored azo dye to estimate nitrite concentration in water samples, a calibration graph must be prepared as described in 2.2.1 to 2.2.4. The bright pink color (azo compound) is developed in solutions with know standard concentrations of nitrite and the intensity of the bright pink color in this series of standard sulutions is evaluated by spectrophotometry at 543 nm. Absorbance values are plotted on the Y-axis versus their respective concentrations of nitrite on the X-axis to give a calibration graph.

3. Nitrate (Parson et al., 1989)

3.1 Outline of method

Nitrate in seawater is reduced almost quantitatively to nitrite when a sample is run through a column containing cadmium filings coated with metallic copper. The nitrite produced is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine to form a highly colored azo dye which can be measured spectrophtometrically. Any nitrite initially present in the sample must be corrected for.

3.2 Experimental procedure

3.2.1 Filter the water sample through Whatman No. 42 filter paper.

3.2.2 Add 20 ml of concentrated ammonium chloride to the sample about 100 ml in the Erlenmeyer flask. Mix the solution and pour about 5 ml onto the top of the column and allow it to pass through.

3.2.3 Add the remainder of the sample to the column and place the drained Erlenmeyer flask under the collection tube. Collect about 40 ml and discard; collect 50 ml in a graduated cylinder and dispense this into the Erlenmeyer flask which contained the original sample. Allow the column to drain before adding the next 5 ml sample.

3.2.4 To the 50 ml sample, add 1.0 ml of sulfanilamide solution from an automatic pipette, mix and allow the reagent to react for a period greater than 2 min but not exceeding 8 min. Add 1 ml of naphthylethylinediamine solution and mix immediately. Between 10 min and 2 hr afterwards, measure the extinction of the solution using a wavelength of 543 nm.

3.2.5 The concentration of nitrate can then be estimated by reference to the calibration graph.

3.2.6 Add about 100 ml of the dilute standard nitrate solution to a 125 ml Erlenmeyer flask and carry out the experimental procedure as described in 3.2.1 to 3.2.4. Absorbance values are plotted on the Y-axis versus their respective concentrations of nitrate on the X-axis to give a calibration graph.

4. Phosphate (Parson et al., 1989)

4.1 Outline of method

The seawater sample is allowed to react with a composite reagent containing molybdic acid, ascorbic acid and trivalent antimony. The resulting complex is reduced to

give a blue solution which is measured at 885 nm. The intensity of the blue color from this solution increases in proportion to the amount of phosphate present since uncomplexed molybdenum in solution does not react with the reducing agent.

4.2 Experimental procedure

4.2.1 Warm the samples to room temperature (15-30 ˚C).

4.2.2 To a 100 ml sample, add 10 ml of mixed reagent using a syringe-type pipette and mix at once.

4.2.3 After 5 min, and preferably within the first 2-3 hr, measure the absorbance using a wavelength of 885 nm.

4.2.4 The concentration of phosphate can then be estimated by reference to the calibration graph.

4.2.5 Add about 100 ml of the dilute standard phospahte solution to a 125 ml Erlenmeyer flask and carry out the experimental procedure as described in 4.2.1 to 4.2.4. Absorbance values are plotted on the Y-axis versus their respective concentrations of phosphate on the X-axis to give a calibration graph.

5. Total phosphorus (Takeuchi, 1988)

5.1 Outline of method

To solubilize all of the phosphorus in a dry matter samples (such as settle solid, soil and algae) for measurement of total phosphorus, all of samples must be digested in boiling perchloric acid. Phosphorus is then measured by the ascorbic acid method. Many

different extracting solutions have been used, including water, salt solutions, dilute hydrochloric and sulfuric acids, and acidic ammonium fluoride solution (Boyd, 1995).

5.2 Reagent

5.2.1 Buffer solution: dissolve 136 g of sodium acetate trihydrate ($CH_3COONa.3H_2O$) in distilled water, add 240 g of acetic acid and dilute to 1000 ml with distilled water.

5.2.2 Ammonim molybdate solution: 2.5 g ammonium molbdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) in 100 ml volumetric flask and add 3 drops of H_2SO_4 conc.

5.2.3 Ascorbic acid solution: 1 g of ascorbic acid/100 ml distilled water.

5.2.4 Nitric acid: concentrated.

5.2.5 Perchloric acid: (70%).

5.2.6 Phosphorus standard solution: (1000 ppm).

5.2.7 Phosphorus standard solution: (20 ppm), use 1 ml of phosphorus standard solution (1 ml = 1 mg) and dilute with distilled water to 50 ml.

5.3 Experimental procedure

A. Digestion

5.3.1 Weight sample and put it in a 250 ml Kjeldahl flask(diet: 0.5 g, meat: 1.0 g).

5.3.2 Add 5-10 ml of HNO_3 then boil until solution becomes 1 ml (about 10 min).

5.3.3 Add 3-4 ml of perchloric acid and heat gently for about 10-15 min and cool.

5.3.4 Add 10 ml of distilled water.

5.3.5 Heat to boiling, cool in room temperature and make up to 50 ml.

5.3.6 Pipette the following sample size into a test tube (diet sample: 0.2 ml).

5.3.7 Add 5 ml of buffer solution.

5.3.8 Add 1 ml of ammonium molybdate solution.

5.3.9 Add 1 ml of ascorbic acid solution.

5.3.10 Make up to 25 ml.

5.3.11 Put the test tube into a water bath of 30-40 ˚C for 40-60 min.

5.3.12 Prepare a sample blank (without sample).

5.3.13 Prepare standard concentration take 3 ml of standard solution (1000 ml). The analytical procedure is the same as that for sample.

5.3.14 Determine the absorbance with a spectrophotometer at 725-750 nm.

B. Standard curve

Pipette the following volumes of the phosphorus standard solution (20 ppm) 0, 0.5, 1, 2, 3 and 4 ml into each test tube and then start from step 5.3.7-5.3.14 (phosphorus content of each tube 0, 10, 20, 40, 60 and 80 ppm, respectively).

C. Calculation

$$P\ (mg/g\ sample) = \frac{(Sample - Blank) \times 1 \times 0.2 \times 25 \times 50}{Standard\ concentration \times Sample\ weight\ (g) \times 1000}$$

6. Total nitrogen (AOAC, 1980)

6.1 Outline of method

The most widely used method employed for determination of protein in organic substances is the Kjeldahl method. It is based on the fact that upon digestion with sulfuric acid and various catalysts, the organic compounds are destroyed and the nitrogen is converted to ammonium sulfate. Upon making the reaction mixture alkaline, ammonia is liberated which is removed by steam distillation, collected and titrated. Total nitrogen was then calculated by divide Kjeldahl protein content by 6.25

6.2 Experimental procedure

6.2.1 Special apparatus and reagent

6.2.1.1 Gerhardt Kjeldatherm disgestion unit, Germany

6.2.1.2 Gerhardf Vapodest 1, Germany

6.2.1.3 Catalyst (Kjel-tab) was contained 3.5 g K_2SO_4 and 0.0035 g Se Tecator, Sweden

6.2.1.4 Indicator was contained 0.625 g of methyl red and 0.480 g of methylene blue which dissolved in ethyl alcohol (50 ml, 95% V/V).

6.2.2 Procedure

6.2.2.1 Weight 2 g of sample in nitrogen free filter paper and put in a 250 ml digestion tube, added 20 ml of conc. sulfuric acid and 1 Kjel-tab.

6.2.2.2 Digest the sample in a digestion unit which was preheated at 200 ˚C for 20 min.

6.6.2.3 Increase the temperature at 20 ˚C for every 20 min until it reached 380 ˚C (completely digested in 3-4 hr).

6.2.2.4 After digestion, the solution was cooled down to room temperature.

6.2.2.5 The digested solution was added with 90 ml of distilled water and distilled with a solution of sodium hydroxide (70 ml, 50% V/V).

6.2.2.6 Ammonia was collected in boric acid (100 ml, 4% w/V). 3-4 drops of indicator was added.

6.2.2.7 Determination of ammonia concentration was performed by titrate with 0.5 N sulfuric acid that was accurately prepared.

6.2.2.8 The volume of sulfuric acid used during titration was recorded and % protein was calculated with the following equation.

$$\% \text{ protein} = (A \times B \times 6.25 \times 1.4)/C$$

where; A = normality of sulfuric acid used to titration

B = ml of sulfuric acid used to titration and

C = weight of sample (g)

7. Alkalinity (APHA, 1992)

7.1 Outline of method

Alkalinity can be defined as the number of equivalents of strong acid required to titrate one litre of water to the CO_2-HCO_3^- endpoint. This is the same as the sum of the concentrations of the anions of carbonic and other weak acids.

7.2 Experimental procedure

7.2.1 Set up a pH meter and burette so that the pH of the sample can be measured as it is being titrated and stirred.

7.2.2 Calibrate the pH meter with pH 4 buffer solution.

7.2.3 Pipette 100 ml of sample into a beaker.

7.2.4 Place the beaker on a magnetic stirrer (use low speed and a small bar). Record the starting pH. Also record the starting level of the H_2SO_4 solution in the burette.

7.2.5 Titrate as rapidly as possible until the pH of the sample is reduced to 4.5. Record the difference between the starting level of H_2SO_4 solution in the burette and the level after titration (total amount of titrant added to the sample).

7.2.6 Calculate alkalinity in mg as $CaCO_3$ per litre by the following equation:

$$\text{mg as } CaCO_3\ l^{-1} = (A \times N \times 50000)/\text{ml of sample}$$

where; A = the average volume of standard acid used, and

N = the normality of standard acid (as 0.02 N of H_2SO_4).

8. Moisture (Boyd, 1995)

8.1 Outline of method

The wet sample is weighted, water is removed by heating at 105 ˚C, and the amount of water removed is determined by weight loss. Results usually are expressed as the percentage of water in a sample in an oven-dry sample (such as settle solid, shrimp carcass, diet, and algae) basis.

8.2 Experimental procedure

8.2.1 Place 5-10 g of wet sample or 1-2 g of air-dried sample in tared weighing containers (tare must include lid weight), closed lids, and weight to the nearest milligram.

8.2.2 Remove lids, place samples in the oven, and dry them to a constant weight. Samples should be dried for at least 24 hr in an oven.

8.2.3 Longer drying time may be needed in a gravity convection oven. Once drying has started, additional wet samples should not be placed in the oven.

8.2.4 Remove samples from the oven, replace lids, and cool to room temperature in a desiccator. Weight the cool samples. Compute the moisture content as follows:

$$\% \text{ moisture} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Final weight} - \text{Tare weight}} \times 100$$

9. Dry weight (Boyd, 1995)

9.1 Experimental procedure

$$\% \text{ dry weight} = 100 - \left[\frac{\text{Initial weight} - \text{Final weight}}{\text{Final weight} - \text{Tare weight}} \times 100\right]$$

$$\% \text{ dry weight} = 100 - \% \text{ moisture}$$

10. Determination of Chlorophll-a (Bennet and Bogorad, 1972; Parson et al, 1989)

10.1 Experimental procedure

10.1.1 The sample contains 10 ml of algal suspension. The sample is either centrifuged for 5 min at 2000 g (3500 rpm) or filtered through a Whatman GF/C filter 25 mm (diameter).

10.1.2 The pellet or filter was extracted with 10 ml methanol (absolute) and ground in a glass tissue homogenizer. Extraction takes place in a water bath, at 70 ˚C for 2 min then the sample was centrifuged.

10.1.3 Optical density was measured at wavelength 630, 647 and 664 nm by spectrophotometer. Calculate the concentrations of Chlorophyll-*a* in the extract using the following equation:

$C_a = 11.85 \times (OD_{664}) - 1.54 \times (OD_{647}) - 0.08 \times (OD_{630})$

Where; C_a = Chlorophll-*a* concentration, mg l^{-1} and

OD_{630}, OD_{647} and OD_{664} = Corrected optical densities (with a 1-cm light path) at the respective wavelengths.

After determining the concentration of pigment in the extract, calculate the amount of pigment per unit volume as follows:

$$\text{Chlorophyll-}a \text{ (mg m}^{-3}) = \frac{C_a \times v}{V \times 10}$$

Where; v = the volume of absolute methanol in ml

V = the volume of sample in litre, and

C*a* = the chlorophyll-*a* which is substituted for C_a in the above equation.

11. Phytoplankton counting technique (APHA, 1992)

11.1 Counting of plankters

Mix the concentrated sample thoroughtly and then pipette 1.0 ml of concentrated sample into the Sedgwick-Rafter counting chamber (S-R couting) and carefully position the cover glass over the chamber without forming an air bubble. This can usually be accomplished by the cover glass diagonally across the chamber and slowly rotating the cover glass as the sample is introduced from the pipette. Place the counting chamber

beneath the microscope, select a random microscope field and identify and count plankters seen within the ocular micrometer grid.

11.2 Calculation

The Sedgwick-Rafter counting chamber contains exactly 1.0 ml (50 mm long × 20 mm wide × 1 mm deep) and has a surface area of 1,000 mm^2. The exact area viewed within the ocular micrometer grid is also known, so the number of organisms counted in a given number of grids can be expanded to the total number of organisms per milliliter of the concentrated sample. This permits calculation of the number of organisms per millilitre of the original sample. The following formula may be used for the calculation:

$$\text{Number of plankters per ml (No. /ml)} = \frac{C \times 1000}{L \times D \times W \times S}$$

Where; C = number of plankter counted

L = length of each grid (S-R cell length), mm

D = depth of grid (S-R cell depth), mm

W = width of a grid (whipple grid image width), mm and

S= number of grid counted.

APPENDIX C

RAW DATA OF CHAPTER III (EFFECT OF SALINITY (0, 15 AND 30 PSU) ON AMMONIUM AND NITRATE UPTAKE KINETICS OF *SPIRULINA*)

Table 1C ammonium uptake of *Spirulina* at 0 psu (Chapter III).

Time (h)	Ammonium concentration (mg NH_4-N L^{-1})					
	2	**4**	**6**	**8**	**10**	**12**
0	1.51	3.51	5.04	6.89	8.59	10.17
0.17	1.30	3.30	4.79	6.58	8.28	9.86
0.5	1.20	3.13	4.57	6.31	7.87	9.39
1	1.12	2.92	4.31	6.07	7.52	8.88
2	1.03	2.73	4.06	5.67	7.03	8.40
4	0.91	2.47	3.71	5.23	6.51	8.01
8	0.82	2.17	3.50	4.82	6.23	7.55
12	0.77	2.03	3.26	4.42	5.99	7.38
16	0.70	1.96	3.10	4.26	5.49	7.13
20	0.67	1.84	3.02	4.12	5.46	6.91
24	0.59	1.76	2.96	4.09	4.92	6.66

Table 2C Ammonium uptake of *Spirulina* at 15 psu (Chapter III).

Time (h)	Ammonium concentration (mg NH_4-N L^{-1})					
	2	**4**	**6**	**8**	**10**	**12**
0	1.32	3.40	5.47	6.80	8.95	10.88
0.17	1.23	3.31	5.23	6.53	8.63	10.53
0.5	1.06	3.01	4.94	6.27	8.30	10.19
1	0.98	2.85	4.64	5.97	7.88	9.91
2	0.92	2.64	4.37	5.73	7.39	9.21
4	0.87	2.45	4.10	5.38	6.82	8.53
8	0.88	2.33	3.85	5.01	6.38	7.96
12	0.80	2.22	3.61	4.81	6.09	7.64
16	0.77	2.01	3.50	4.58	5.79	7.33
20	0.72	2.00	3.35	4.40	5.61	7.15
24	0.68	1.92	3.27	4.26	5.54	6.93

Table 3C Ammonium uptake of *Spirulina* at 30 psu (Chapter III).

Time (h)	Ammonium concentration (mg NH_4-N L^{-1})					
	2	**4**	**6**	**8**	**10**	**12**
0	1.75	3.73	5.33	6.86	8.65	10.75
0	1.63	3.48	5.05	6.51	8.40	10.42
1	1.39	3.13	4.63	6.11	8.01	9.92
1	1.29	2.97	4.41	5.94	7.50	9.61
2	1.20	2.77	4.24	5.66	7.16	9.17
4	1.15	2.65	4.04	5.41	6.88	8.79
8	1.06	2.52	3.88	5.10	6.57	8.54
12	0.99	2.33	3.72	5.04	6.33	8.28
16	0.93	2.22	3.56	4.69	6.18	8.05
20	0.88	2.10	3.39	4.52	5.91	7.59
24	0.85	2.01	3.35	4.41	5.67	7.39

Table 4C *Spirulina* chlorophyll-a concentration of the ammonium uptake study (Chapter III).

Salinity (psu)	Chl-*a* (mg Chl-*a* L^{-1})					
	2 mg NH_4-N L^{-1}	**4 mg NH_4-N L^{-1}**	**6 mg NH_4-N L^{-1}**	**8 mg NH_4-N L^{-1}**	**10 mg NH_4-N L^{-1}**	**12 mg NH_4-N L^{-1}**
0	2.82	5.75	4.77	4.77	5.84	5.00
15	7.41	7.14	7.09	6.39	7.23	7.09
30	12.88	13.02	12.32	11.49	10.75	14.27

Table 5C Nitrate uptake of *Spirulina* at 0 psu (Chapter III).

Time (h)	Nitrate concentration (mg NO_3-N L^{-1})				
	5.13	**10.48**	**20.64**	**5.18**	**98.37**
0	5.13	10.48	20.64	50.18	98.37
0.17	4.83	10.40	20.13	49.05	97.19
0.5	4.59	9.19	19.13	48.59	95.04
1	4.02	8.43	18.00	45.74	94.28
2	3.67	8.19	16.79	45.53	91.17
4	3.57	7.49	16.87	45.13	90.74
8	3.41	6.85	16.12	44.88	90.09
12	3.08	6.71	15.91	44.05	89.82
16	2.98	6.34	15.10	43.86	89.18
20	2.87	5.74	14.99	42.76	88.75
24	2.73	5.45	14.13	42.25	88.16

Table 6C Nitrate uptake of *Spirulina* at 15 psu (Chapter III).

Time (h)	Nitrate concentration (mg NO_3-N L^{-1})				
	6.05	**9.92**	**19.75**	**47.15**	**102.5**
0	6.05	9.92	19.75	47.15	102.50
0.17	6.00	9.84	19.28	47.04	102.11
0.5	5.62	8.72	18.63	46.20	101.88
1	5.10	8.01	17.24	45.40	100.10
2	4.90	7.65	16.80	44.98	99.96
4	4.88	7.54	16.86	44.86	99.22
8	4.84	7.44	16.28	44.77	98.98
12	4.71	7.39	16.11	44.14	98.44
16	4.65	7.02	15.94	43.90	97.84
20	4.50	6.85	15.90	43.55	97.03
24	4.29	6.70	15.85	42.12	96.75

Table 7C Nitrate uptake of *Spirulina* at 30 psu (Chapter III).

Time (h)	Nitrate concentration (mg NO_3-N L^{-1})				
	4.84	**9.26**	**19.98**	**47.63**	**91.28**
0	4.84	9.26	19.98	47.63	91.28
0.17	4.77	8.96	19.95	47.25	91.15
0.5	4.39	8.81	19.46	47.10	90.95
1	4.22	8.56	19.14	46.65	89.91
2	3.80	7.87	18.12	45.64	87.80
4	3.68	7.52	17.74	45.24	87.03
8	3.58	7.30	17.62	44.62	86.32
12	3.53	7.18	17.52	44.20	85.96
16	3.48	6.80	17.35	44.08	85.45
20	3.40	6.56	17.28	43.23	85.29
24	3.35	6.33	16.14	42.66	84.95

Table 8C *Spirulina* chlorophyll-a concentration of the nitrate uptake study (Chapter III).

Salinity (psu)	Chl-*a* (mg Chl-*a* L^{-1})				
	5 mg NO_3-N L^{-1}	10 mg NO_3-N L^{-1}	20 mg NO_3-N L^{-1}	50 mg NO_3-N L^{-1}	100 mg NO_3-N L^{-1}
0	0.79	1.34	3.18	7.03	14.95
15	6.72	7.14	9.64	7.37	7.65
30	5.98	6.07	5.65	4.96	7.09

APPENDIX D

RAW DATA OF CHAPTER IV (WATER QUALITY CONTROL USING *Spirulina platensis* IN SHRIMP CULTURE TANKS)

Table 1D ammonium, nitrite and nitrate concentrations in water of Trial I (Chapter IV).

Day	Control (mg-N L^{-1})			Treatment 1 (mg -N L^{-1})			Treatment 2 (mg -N L^{-1})		
	NH_4^+	NO_2^-	NO_3^-	NH_4^+	NO_2^-	NO_3^-	NH_4^+	NO_2^-	NO_3^-
1	0.025	0.008	0.736	0.358	0.096	8.143	0.364	0.104	8.164
3	0.036	0.024	0.863	0.318	0.086	7.174	0.327	0.079	7.461
5	0.137	0.061	0.906	0.249	0.070	5.354	0.250	0.067	5.440
7	0.207	0.103	1.403	0.114	0.061	4.225	0.150	0.036	4.284
9	0.304	0.139	2.434	0.052	0.120	2.334	0.091	0.048	5.008
11	0.414	0.213	4.377	0.062	0.140	5.337	0.090	0.065	5.517
13	0.352	0.254	8.247	0.177	0.068	9.746	0.062	0.041	8.895
14	0.341	0.250	10.496	0.138	0.044	12.465	0.055	0.020	13.157
16	0.261	0.242	12.223	0.101	0.036	10.309	0.042	0.019	12.774
18	0.260	0.288	12.720	0.088	0.068	8.968	0.032	0.032	7.953
20	0.284	0.333	13.456	0.024	0.147	6.120	0.028	0.056	4.155
22	0.312	0.384	14.733	0.023	0.245	5.210	0.021	0.087	3.582
24	0.329	0.412	15.375	0.017	0.295	4.291	0.022	0.120	3.521
26	0.353	0.393	16.017	0.040	0.268	6.269	0.012	0.125	4.305
28	0.377	0.396	16.232	0.044	0.233	10.343	0.015	0.142	4.406
30	0.382	0.389	15.809	0.054	0.218	14.018	0.031	0.144	4.546
32	0.402	0.407	15.367	0.060	0.221	15.218	0.038	0.146	4.931
34	0.383	0.431	15.346	0.114	0.302	11.401	0.010	0.149	5.038
36	0.429	0.443	15.337	0.129	0.334	6.815	0.007	0.144	5.241
38	0.456	0.456	15.324	0.085	0.345	7.963	0.008	0.134	5.376
40	0.489	0.496	15.830	0.063	0.341	6.860	0.007	0.132	5.142
42	0.508	0.498	16.173	0.047	0.350	5.954	0.002	0.132	4.564
44	0.515	0.501	16.571	0.030	0.331	5.121	0.001	0.130	4.206

Table 2D Chlorophyll-*a* concentration in Trial I (Chapter IV).

Day	Chl-*a* (mg L^{-1})		
	Control	**Treatment 1**	**Treatment 2**
1	0.000	0.015	0.017
1	0.000	0.015	0.017
3	0.000	0.018	0.020
3	0.000	0.018	0.020
5	0.000	0.025	0.034
5	0.000	0.025	0.020
7	0.005	0.067	0.066
7	0.005	0.067	0.029
9	0.003	0.060	0.051
9	0.003	0.060	0.022
11	0.009	0.034	0.038
11	0.009	0.034	0.016
13	0.007	0.029	0.016
13	0.007	0.029	0.012
14	0.010	0.013	0.013
14	0.010	0.013	0.013
16	0.011	0.015	0.010
16	0.011	0.015	0.010
18	0.013	0.024	0.011
18	0.013	0.024	0.011
20	0.015	0.054	0.017
20	0.015	0.054	0.013
22	0.016	0.066	0.027
22	0.016	0.066	0.018
24	0.014	0.076	0.035
24	0.014	0.076	0.019
26	0.012	0.069	0.034
26	0.012	0.069	0.017
28	0.013	0.050	0.031
28	0.013	0.050	0.022
30	0.012	0.036	0.036
30	0.012	0.036	0.025
32	0.014	0.028	0.048
32	0.014	0.028	0.030
34	0.008	0.050	0.071
34	0.008	0.050	0.042
36	0.006	0.073	0.081
36	0.006	0.073	0.042
38	0.006	0.066	0.083
38	0.006	0.066	0.057
40	0.008	0.090	0.106
40	0.008	0.090	0.059
42	0.007	0.121	0.110
42	0.007	0.121	0.060
44	0.008	0.132	0.085
44	0.008	0.132	0.047

Table 3D shrimp weight (g) and survival (number of shrimp) of control, treatment 1 and treatment 2 in Trial I (Chapter IV).

Day	Shrimp weight (g)		
	Control	**Treatment 1**	**Treatment 2**
0	4.3	4.4	4.4
0	4.2	5.9	5
0	4	3.9	4.5
0	4.6	4	4.5
0	4.1	4.4	4.2
0	4.3	3.8	4
0	5.4	4.4	4.5
0	4.8	3.8	4
0	4.1	3.8	4.2
0	4.8	4.2	3.9
0	5	4.2	4.2
0	5.2	4.2	4.1
0	5.5	4.7	5.3
0	4.5	4.1	5.3
0	4.5	4.7	4.5
0	5.1	4.8	4.4
0	4.2	4.2	5.3
0	3.9	4	5.6
0	5.2	5.3	4.3
0	3.9	4.6	4.8
0	4.2	5.5	5
0	4.7	5.1	4.9
0	5.3	4.8	4.9
0	4	4.7	4.7
0	4.7	5	5.5
0	5.7	4.6	4.3
0	4.8	4.3	4.9
0	4.2	5.3	4.5
0	5	5.1	5.9
0	4	4.6	4
15	4.2	4.8	3.6
15	6.3	5	5.1
15	5	5.7	6.3
15	5.3	5.3	4.9
15	5.3	5.6	6.4
15	5.2	5.1	4.1
15	4.6	5.2	5.3
15	5.3	5	4.4
15	5.4	4.7	5.8
15	4.5	6.3	6
15	5.3	6.2	5
15	5.3	4.9	4.2
15	5.5	6.1	6.3
15	5.6	5.7	6.8
15	5.2	5	5.2
15	4.4	5.8	6.4
15	5.6	5.6	5.7
15	4.7	4.6	5.5
15	4.8	6.1	5.4
15	4.7	5.1	6.2

Table 3D (Continued)

Day	Shrimp weight (g)		
	Control	**Treatment 1**	**Treatment 2**
15	4.7	6.1	6.3
15	4.7	5.7	5.2
15	5	6.1	7.2
15	7.5	5.7	5.1
15	6.7	6.9	6
15	5.5	6.2	4.2
15	5.2	6.4	4.3
15	5.7	5.6	4.8
15	5.5	5.7	6
15	6.6	5	5.2
30	6.6	5.9	4
30	4.7	7.1	6.8
30	5.5	4.7	7.1
30	5.7	5.6	6.8
30	5	6.6	6.5
30	5.6	5.7	5.8
30	4.8	8.2	7
30	5	6.5	8.6
30	6.1	6.7	5.3
30	4.6	6.2	6.9
30	5	5.4	7.3
30	5.6	5.7	8.6
30	7.1	7.7	5.9
30	4.8	6.1	6.4
30	4.8	6.1	6.6
30	5.2	6.1	4.6
30	8.4	6.6	6.5
30	6.5	6.8	8.3
45	4.4	4.9	6.6
45	5.9	5.8	6.3
45	5.4	7	7.2
45	5.9	6.6	7
45	5.8	5.6	3.8
45	7.2	6.1	6.60
45	4.5	7.3	6.3
45	5.5	6.3	5.6
45	5.6	5.8	6.6
45	5.3	7.8	8
45	6.1	6.7	6.8
45	5.2	6.2	9
45	5.1	7.6	6.1
45	4.7	-	4.1
45	7.4	-	4.5
45	7.1	-	6

Table 4D Number of *Spirulina* (trichomes mL^{-1}) of treatment 1 and 2 in Trial I (Chapter IV).

Day	Number of *Spirulina* (trichomes mL^{-1})	
	Treatment 1	**Treatment 2**
1	516.67	416.67
1		416.67
3	610.00	393.33
3		393.33
5	826.67	670.00
5		403.33
7	1596.67	1190.00
7		643.33
9	1426.67	826.67
9		370.00
11	816.67	603.33
11		273.33
13	783.33	480.00
13		293.33
14	460.00	290.00
14		290.00
16	576.67	300.00
16		300.00
18	760.00	346.67
18		346.67
20	1046.67	486.67
20		353.33
22	1233.33	666.67
22	-	403.33
24	1476.67	810.00
24	-	390.00
26	1656.67	730.00
26	-	353.33
28	1073.33	626.67
28	-	396.67
30	766.67	766.67
30	-	496.67
32	676.67	953.33
32	-	610.00
34	703.33	1103.33
34	-	490.00
36	783.33	1073.33
36	-	513.33
38	753.33	1190.00
38	-	796.67
40	810.00	1153.33
40	-	563.33
42	1223.33	943.33
42	-	526.67
44	1453.33	840.00
44	-	516.67

Table 5D ammonium, nitrite and nitrate concentrations in water of Trial II (Chapter IV).

Day	Control 1 (mg-N L^{-1})			Treatment 1 (mg-N L^{-1})		
	NH_4^+	NO_2^-	NO_3^-	NH_4^+	NO_2^-	NO_3^-
1	0.037	0.022	0.301	0.309	0.092	6.102
4	0.054	0.024	0.125	0.301	0.089	6.063
6	0.092	0.032	0.125	0.250	0.077	5.471
10	0.276	0.100	0.906	0.190	0.057	4.311
13	0.306	0.172	1.898	0.101	0.048	3.003
15	0.297	0.211	4.503	0.048	0.040	2.852
16	0.280	0.224	6.205	0.035	0.040	2.305
19	0.254	0.224	7.401	0.035	0.025	2.105
21	0.249	0.224	7.608	0.040	0.067	3.003
23	0.230	0.220	7.603	0.030	0.078	3.801
25	0.230	0.205	8.605	0.043	0.085	5.801
27	0.220	0.197	9.205	0.046	0.096	5.704
29	0.225	0.205	9.703	0.035	0.096	5.404
31	0.216	0.235	9.002	0.035	0.096	4.533
33	0.227	0.254	9.503	0.010	0.077	4.401
35	0.246	0.249	9.704	0.000	0.060	3.104
37	0.265	0.249	9.703	0.000	0.033	2.406
39	0.257	0.235	10.903	0.011	0.060	2.003
41	0.262	0.249	11.809	0.014	0.043	2.300
43	0.270	0.246	12.604	0.002	0.043	1.801
45	0.284	0.260	12.904	0.009	0.043	1.808
47	0.308	0.292	13.103	0.023	0.012	1.603
51	0.320	0.300	13.603	0.010	0.007	1.180
53	0.333	0.311	14.303	0.000	0.007	1.405
56	0.368	0.335	15.105	0.007	0.009	1.302
58	0.390	0.354	15.602	0.000	0.007	1.304
60	0.427	0.373	16.006	0.000	0.002	1.403

Table 5D (Continued)

Day	Control 2 (mg-N L^{-1})			Treatment 2 (mg-N L^{-1})		
	NH_4^+	NO_2^-	NO_3^-	NH_4^+	NO_2^-	NO_3^-
1	0.040	0.019	0.425	0.330	0.097	7.101
4	0.128	0.028	0.481	0.308	0.088	6.303
6	0.303	0.069	0.723	0.246	0.071	5.802
10	0.393	0.215	2.504	0.102	0.065	5.503
13	0.374	0.328	6.410	0.061	0.034	4.304
15	0.328	0.348	8.005	0.019	0.039	2.613
16	0.313	0.367	9.002	0.017	0.034	2.005
19	0.303	0.348	12.725	0.013	0.014	1.342
21	0.298	0.313	13.448	0.036	0.014	1.011
23	0.294	0.298	13.801	0.054	0.028	1.853
25	0.294	0.287	13.450	0.057	0.060	2.704
27	0.293	0.294	14.525	0.053	0.081	3.704
29	0.298	0.298	13.801	0.050	0.090	4.401
31	0.338	0.287	13.566	0.032	0.081	5.083
33	0.376	0.290	13.788	0.018	0.056	5.103
35	0.372	0.300	14.886	0.004	0.043	4.005
37	0.343	0.352	15.003	0.010	0.025	3.202
39	0.352	0.380	15.124	0.005	0.020	3.534
41	0.375	0.380	16.089	0.005	0.014	3.245
43	0.380	0.363	18.845	0.010	0.023	3.433
45	0.382	0.390	19.925	0.008	0.029	3.924
47	0.397	0.424	19.923	0.010	0.025	4.116
51	0.394	0.428	20.406	0.010	0.025	3.124
53	0.417	0.432	21.000	0.008	0.032	3.110
56	0.443	0.443	21.124	0.009	0.028	2.701
58	0.478	0.451	22.447	0.002	0.015	2.199
60	0.524	0.487	22.929	0.010	0.013	2.205

Table 6D Chlorophyll-*a* concentration in Trial II (Chapter IV).

Table 7D shrimp weight (g) and survival (number of shrimp) of control 1, Control 2, treatment 1 and treatment 2 in Trial II (Chapter IV)

Day	Shrimp weight (g)			
	Control 1	Control 2	Treatment 1	Treatment 2
0	3.7	4.1	3.7	4.3
0	2.4	3.3	3.6	3.5
0	2.5	3.2	3.8	3.4
0	3.4	2.5	3.8	3.5
0	4.1	3.1	4.2	3.5
0	2.5	4.1	3.5	2.2
0	3.9	3.7	3.9	3.2
0	2.8	3.8	4.1	4.3
0	2.4	2.3	4.4	2.5
0	2.8	4.2	3.4	2.7
0	3.3	3.1	3.5	3.8
0	2.2	4	4.4	2.5
0	2.3	2.7	3.4	2.9
0	4.2	2.8	3.4	2.7
0	2.4	1.8	3.8	3.9
0	2.2	2.9	3.3	2.5
0	2.8	2.9	4.7	2.8
0	2.8	2.9	3.4	3.2
0	2.7	3.6	4.4	3.3
0	3.1	4.4	4.3	3.3
0	2	4.3	3.9	3.2
0	3.2	4.5	2.8	2.7
0	1.7	3	3.2	3.2
0	3.5	3.1	2.8	2.7
0	2.8	4.4	-	-
0	3.2	3.4	-	-
0	2	3.3	-	-
0	2	4.5	-	-
0	3.7	3.6	-	-
0	4.4	4.3	-	-
0	2.2	2.9	-	-
0	2	3.5	-	-
0	2.6	2.8	-	-
0	2.6	4.5	-	-
0	2.4	2.8	-	-
0	2.2	4	-	-
0	2.9	3.2	-	-
0	2.6	3.2	-	-
0	2.6	2.9	-	-
0	2.5	2.9	-	-
0	4	3.5	-	-
0	4.2	2.8	-	-
0	3.7	2.5	-	-
0	3.3	3.8	-	-
0	2.4	4	-	-
15	2.7	2.5	3.3	4.1
15	2.9	4	4	4.6
15	2.6	1.9	4.5	2.2
15	3.5	4	4.8	3.4
15	3.3	3.4	4	5
15	3.6	5	4.9	4.2
15	4.1	3.6	4.4	3.3
15	2.6	2.3	3.6	4
15	2.8	3.9	4.1	2.7
15	2.5	3	3.7	3.9
15	4.3	4.1	4.3	3.2
15	4.1	4.2	4.8	2.9
15	2.5	4.9	4.4	3.1

Table 7D (Continue)

Day	Shrimp weight (g)			
	Control 1	Control 2	Treatment 1	Treatment 2
15	3.1	3.3	4.7	2.7
15	3.6	4.6	3.6	3.5
15	3.8	4	3.5	3.9
15	2.5	4.2	4.6	3.6
15	3.5	4.6	3.6	4
15	1.9	4.3	4.5	4
15	4.4	3.4	3.5	4.4
15	3.2	4.6	5.2	3.2
15	2.6	3	3.4	3.5
15	4.1	4.2	4.8	3.1
15	2.7	3	-	-
15	3.2	4.2	-	-
15	2	3.4	-	-
15	2.5	5	-	-
15	3.3	4.6	-	-
15	2.9	4.2	-	-
15	4.4	3.1	-	-
15	4.9	3.9	-	-
15	3.9	3	-	-
15	2.2	3	-	-
15	3.3	3.1	-	-
15	2.9	3.2	-	-
15	3.8	3.8	-	-
15	2.9	2.5	-	-
15	3.8	3.3	-	-
15	2.5	3.1	-	-
15	3		-	-
30	2.9	3.8	4.8	4.6
30	3.3	4.6	5	5.6
30	3.6	6	5.5	5.4
30	2.7	4	3.7	3.8
30	3.3	2.1	5.5	3.7
30	2.7	2.6	4.5	2.7
30	4.7	3.4	4.6	3.2
30	5	3	3.8	4.2
30	4.8	4.1	5.8	3.5
30	3.4	4.5	4.1	3.7
30	4.6	5.1	4.3	3.9
30	2.9	4.9	5.2	3.6
30	2.2	2.9	5.1	4.7
30	2.7	2.4	4.5	3.1
30	2.7	2.9	5.1	4
30	3.7	4.5	5.8	4.4
30	4.2	3.4	3.9	5.3
30	3.9	-	5.7	-
30	2.7	-	6.6	-
30	4.8	-	-	-
30	4	-	-	-
30	2.3	-	-	-
30	5.9	-	-	-
45	3.5	2.1	6.4	4.5
45	3.5	4.4	4.9	3.9
45	3.2	3.5	5.6	5.8
45	3.5	6.2	4.6	4.1
45	3.7	2.7	4.7	4.9
45	2.6	4.5	3.8	3.7
45	5.3	4.9	5.1	4.7
45	3	5.6	3.8	3.5
45	3.4	2.5	4.3	5.2

Table 7D (Continue)

Day	Shrimp weight (g)			
	Control 1	**Control 1**	**Treatment 1**	**Treatment 2**
45	3.2	-	4.2	5.4
45	2.2	-	6	3.5
45	2.6	-	6.1	5
45	5.6	-	5.8	3.3
45	4.9	-	5.7	-
45	4.6	-	3.9	-
45	5.2	-	7.6	-
45	2.5	-	4.6	-
45	5.4	-	-	-
45	4.1	-	-	-
45	4.6	-	-	-
60	2.8	6.7	7.6	5.2
60	4.4	2	4.2	6.2
60	3.9	2.8	5.3	4.4
60	4.1	5.8	4	6.7
60	6.1	6.7	4.5	3.3
60	3.5	5.5	5	5.1
60	6.8	2.7	4.4	3.7
60	3.1	-	9	5.6
60	2.5	-	4.6	6.5
60	3.2	-	5.9	5.3
60	5.7	-	8.4	3.8
60	2.5	-	3.5	4.3
60	5.9	-	6.7	-
60	5.6	-	5.8	-
60	2.5	-	5.9	-
60	4.2	-	-	-
60	5.9	-	-	-
60	6.1	-	-	-

APPENDIX E

RAW DATA OFCHAPTER V (WATER QUALITY AND NUTRIENT BUDGET IN INTEGRATED SHRIMP CULTURE WITH *S. platensis* UNDER OUTDOOR CONDITION)

Table 1E Ammonium, nitrite and nitrate concentrations in water (Chapter V).

Day	Control (mg-N L^{-1})			Treatment 1 (mg-N L^{-1})			Treatment 2 (mg-N L^{-1})		
	NH_4^+	NO_2^-	NO_3^-	NH_4^+	NO_2^-	NO_3^-	NH_4^+	NO_2^-	NO_3^-
1	0.011	0.000	0.393	0.000	0.000	0.295	0.011	0.005	0.336
3	2.281	0.525	0.983	3.124	0.008	0.290	3.489	0.080	0.147
6	6.357	0.192	0.465	5.895	0.109	0.272	6.735	0.321	0.290
10	4.859	1.864	2.557	2.832	1.879	2.716	1.538	1.694	2.390
13	0.138	16.270	20.598	0.000	9.084	12.667	0.012	13.300	17.285
15	0.153	18.947	23.654	0.065	10.348	13.877	0.369	14.476	19.234
17	0.177	21.567	24.103	0.107	12.456	17.918	0.142	15.463	19.501
20	0.153	20.533	27.057	0.158	9.741	21.388	0.185	12.251	17.462
22	0.553	12.803	24.636	0.703	13.295	22.903	0.690	10.468	16.067
24	0.109	18.707	23.757	0.211	16.017	23.388	0.192	11.717	14.149
27	0.435	24.453	30.036	0.067	21.286	28.739	0.172	10.382	12.539
29	0.097	29.116	30.606	0.242	23.474	26.470	0.145	10.669	11.049
31	0.201	30.380	29.739	0.000	25.687	27.521	0.051	9.787	8.801
34	0.177	26.831	35.624	0.102	21.401	31.780	0.316	6.798	9.001
36	0.314	24.575	36.888	0.174	18.149	27.434	0.437	6.256	7.808
38	0.139	25.634	32.967	0.515	15.584	24.026	0.590	7.050	8.031
41	0.524	24.685	34.165	0.378	19.165	26.536	0.265	7.507	8.953
43	0.681	24.925	35.780	0.493	18.471	24.107	0.347	9.210	7.472
45	1.087	27.977	35.934	0.418	17.187	17.934	0.453	9.115	8.453
48	0.426	19.525	28.347	0.585	15.384	19.949	0.646	8.750	8.667
50	0.356	20.034	30.552	0.378	12.397	12.231	0.638	7.741	5.321
52	0.399	21.488	31.616	0.579	10.604	10.334	0.606	6.842	3.603
55	0.192	22.234	34.147	0.299	9.816	10.224	0.350	7.114	3.775
57	0.605	23.488	38.467	0.323	8.423	7.839	0.385	6.724	3.429

Table 2E Phosphate concentrations in water (Chapter V).

Day	Phosphate (mg-P L^{-1})		
	Control	**Treatment 1**	**Treatment 2**
1	0.057	0.049	0.065
3	0.358	0.498	0.603
6	0.743	0.784	0.902
10	1.345	0.874	1.517
13	1.190	0.972	1.734
15	1.336	0.716	2.550
17	1.517	1.080	2.478
20	1.860	1.787	2.813
22	2.116	2.218	3.058
24	2.364	3.010	3.217
27	2.692	3.664	3.661
29	3.538	4.076	4.232
31	3.812	4.197	4.423
34	4.495	4.024	4.560
36	4.576	3.631	4.751
38	4.893	3.157	5.058
41	5.475	3.339	5.431
43	5.695	4.376	5.998
45	5.780	4.535	5.932
48	5.711	5.854	6.113
50	6.188	6.010	6.161
52	6.506	6.113	6.430
55	6.952	6.548	6.756
57	7.135	6.371	6.993

Table 3E pH and temperature (°C) in water (Chapter V).

Time	Control		Treatment 1		Treatment 2	
	pH	Temperature	pH	Temperature	pH	Temperature
0:00	8.54	27.65	8.80	26.50	8.49	27.55
0:30	8.54	27.60	8.79	26.40	8.51	27.55
1:00	8.54	27.55	8.77	26.30	8.50	27.45
1:30	8.54	27.50	8.77	26.25	8.51	27.45
2:00	8.53	27.50	8.77	26.20	8.50	27.40
2:30	8.53	27.40	8.76	26.10	8.50	27.35
3:00	8.54	27.35	8.76	26.05	8.50	27.25
3:30	8.54	27.35	8.75	25.95	8.49	27.25
4:00	8.54	27.30	8.75	25.90	8.49	27.15
4:30	8.54	27.25	8.75	25.85	8.48	27.10
5:00	8.54	27.20	8.74	25.75	8.48	27.10
5:30	8.53	27.15	8.75	25.65	8.48	27.00
6:00	8.55	27.05	8.75	25.60	8.48	27.00
6:30	8.55	27.00	8.74	25.50	8.48	26.90
7:00	8.56	26.95	8.73	25.45	8.48	26.90
7:30	8.57	26.90	8.74	25.40	8.48	26.80
8:00	8.57	26.85	8.74	25.40	8.49	26.80
8:30	8.58	26.85	8.75	25.35	8.51	26.75
9:00	8.60	26.85	8.76	25.30	8.54	26.70
9:30	8.62	26.80	8.77	25.30	8.54	26.65
10:00	8.64	26.95	8.81	25.50	8.54	26.70
10:30	8.71	27.30	8.85	25.80	8.55	26.70
11:00	8.77	27.65	8.90	26.20	8.56	26.70
11:30	8.82	28.00	8.95	26.65	8.59	26.75
12:00	8.87	28.35	9.00	27.15	8.61	26.85
12:30	8.88	28.60	9.04	27.60	8.63	27.10
13:00	8.91	28.75	9.08	28.05	8.67	27.20
13:30	8.91	28.85	9.12	27.65	8.68	27.30
14:00	8.88	28.90	9.10	27.80	8.69	27.35
14:30	8.87	28.95	9.10	27.75	8.79	27.35
15:00	8.77	30.15	9.09	27.75	8.89	28.25
15:30	8.78	29.00	9.08	27.70	8.89	28.30
15:30	8.83	28.30	9.07	27.70	8.87	28.30
16:00	8.81	28.35	9.06	27.55	8.85	28.30
16:30	8.80	28.30	9.03	27.55	8.82	28.25
17:00	8.77	28.25	9.00	27.45	8.78	28.25
17:30	8.74	28.20	8.97	27.40	8.75	28.20
18:00	8.70	28.15	8.95	27.30	8.73	28.15
18:30	8.68	28.05	8.93	27.20	8.69	28.10
19:00	8.66	28.05	8.91	27.15	8.65	28.00
19:30	8.63	28.00	8.89	27.05	8.62	28.00
20:00	8.62	28.00	8.87	27.00	8.60	27.90
20:30	8.60	27.95	8.86	26.95	8.57	27.90
21:00	8.59	27.90	8.84	26.85	8.55	27.80
21:30	8.58	27.90	8.83	26.75	8.54	27.80
22:00	8.56	27.85	8.82	26.70	8.52	27.70
22:30	8.57	27.75	8.81	26.60	8.52	27.70
23:00	8.56	27.75	8.81	26.55	8.50	27.65

Table 4E Average alkalinity concentrations in water (Chapter V).

Day	Average alkalinity (mg-$CaCO_3$ L^{-1})		
	Control	**Treatment 1**	**Treatment 2**
1	194.0	195.5	194.5
3	211.0	221.5	219.0
6	223.0	223.0	225.0
14	167.5	158.0	167.5
22	147.5	131.0	138.5
28	146.0	127.0	163.5
37	148.0	146.0	179.0
45	139.5	158.0	179.0
50	149.0	177.5	177.5

Table 5E Chlorophyll-*a* concentration (Chapter V).

Day	Chl-*a* (mg L^{-1})		
	Control	**Treatment 1**	**Treatment 2**
13	0.017	0.078	0.003
15	0.017	0.071	0.003
17	0.020	0.075	0.002
20	0.021	0.049	0.001
22	0.024	0.044	0.001
24	0.039	0.038	0.001
27	0.049	0.036	0.005
29	0.052	0.037	0.003
31	0.045	0.033	0.002
34	0.038	0.039	0.002
36	0.030	0.047	0.002
36	0.030	0.035	0.002
37	-	0.046	-
37	-	0.031	-
38	0.026	0.033	0.002
38	0.025	0.023	0.002
41	0.027	0.025	0.004
41	0.027	0.018	0.004
43	0.012	0.018	0.002
45	0.035	0.017	0.007
48	0.029	0.022	0.003
50	0.028	0.028	0.003
52	0.027	0.034	0.002
52	0.027	0.027	0.002
55	0.025	0.033	0.004
55	0.025	0.029	0.004
56	-	0.028	-
56	-	0.020	-
57	0.021	0.024	0.005
57	0.021	0.022	0.005

Table 6E Number of *Spirulina* (trichomes mL^{-1}) of treatment 1 and 2 (Chapter V).

Day	Number of *Spirulina* (trichomes mL^{-1})	
	Treatment 1	**Treatment 2**
1	28.13	162.50
2	0.00	193.75
3	12.50	206.25
4	15.63	203.13
6	40.63	153.13
7	46.88	90.63
8	68.75	68.75
10	106.25	128.13
13	356.25	446.88
15	421.88	337.50
17	465.63	446.88
20	531.25	371.88
22	1068.75	212.50
24	1706.25	243.75
27	2637.50	237.50
29	3359.38	387.50
31	4337.50	362.50
34	6262.50	437.50
36	7768.75	397.32
36	6693.75	397.32
37	7768.75	437.50
37	6668.75	437.50
38	6867.71	906.25
38	6462.50	906.25
41	5481.25	1150.00
41	4987.50	1150.00
43	4018.75	1062.50
45	3693.75	968.75
48	4237.50	868.75
50	4906.25	800.00
52	6906.25	862.50
52	6293.75	862.50
55	7306.25	962.50
55	6206.25	962.50
56	6481.25	1018.75
56	5256.25	1018.75
57	5531.25	1050.00
57	4962.50	1050.00

Table 7E Estimated nitrogen accumulation from feed and total inorganic nitrogen concentration in water of control, treatment 1 and 2 (Chapter V).

Day	Accumulated nitrogen from feed (g-N Tank^{-1})			Total inorganic nitrogen in water (g-N Tank^{-1})		
	Control	Treatment 1	Treatment 2	Control	Treatment 1	Treatment 2
1	13.25	13.28	12.92	0.18	0.12	0.16
3	39.75	39.85	38.76	1.82	1.64	1.78
6	79.50	79.70	77.52	3.37	3.01	3.53
10	132.51	132.83	129.20	4.45	3.56	2.70
13	172.26	172.68	167.97	17.76	10.43	14.68
15	198.76	199.25	193.81	20.52	11.66	16.35
17	225.26	225.81	219.65	22.01	14.63	16.85
20	265.02	265.66	258.41	22.92	15.02	14.35
22	291.52	292.23	284.25	18.24	17.71	13.07
24	318.02	318.79	310.09	20.43	19.02	12.51
27	357.77	358.64	348.85	26.36	24.04	11.08
29	384.27	385.21	374.69	28.71	24.09	10.49
31	410.77	411.77	400.53	28.95	25.53	8.95
34	450.53	451.62	439.29	30.06	25.58	7.74
36	477.03	478.19	465.13	29.65	21.96	6.96
38	503.53	504.75	490.98	28.20	19.26	7.52
41	543.28	544.60	529.74	28.50	22.12	8.03
43	569.78	571.17	555.58	29.47	20.67	8.17
45	596.29	597.74	581.42	31.20	17.06	8.65
48	636.04	637.58	620.18	23.18	17.24	8.67
50	662.54	664.15	646.02	24.45	12.00	6.45
52	689.04	690.72	671.86	25.68	10.33	5.15
55	728.79	730.57	710.62	26.90	10.28	5.39
57	755.30	757.13	736.46	28.97	9.13	5.22

Table 8E Shrimp weight (g) and survival (number of shrimp) of control , treatment 1 and treatment 2 (Chapter V).

Day	Shrimp weight (g)		
	Control	Treatment 1	Treatment 2
0	6.28	5.08	3.81
0	6.26	4.30	4.57
0	4.66	5.02	4.24
0	4.73	4.62	5.13
0	6.50	4.25	6.59
0	6.86	3.40	5.63
0	4.33	5.19	5.75
0	4.48	4.65	4.95
0	4.38	4.62	4.05
0	4.62	5.38	5.57
0	4.28	6.23	5.67
0	4.85	4.49	5.27
0	3.42	3.05	3.87
0	3.69	3.53	3.50
0	4.90	5.58	3.55
0	3.58	4.48	5.45
0	4.07	4.01	3.47
0	4.58	4.04	3.40
0	3.74	3.69	3.22
0	5.52	4.91	3.73
0	4.16	3.97	4.44
0	3.68	4.16	6.21
0	6.13	5.53	4.85
0	5.25	5.47	4.97
0	3.96	5.06	3.21
0	5.26	4.93	3.52
0	5.83	3.65	2.93
0	5.67	4.78	4.08
0	4.74	4.23	4.83
0	5.79	5.42	5.94
0	6.57	3.80	3.56
0	5.20	5.80	6.29
0	4.48	5.48	5.33
0	4.47	6.27	5.30
0	6.15	5.54	3.58
0	4.13	6.17	3.86
0	4.54	4.84	3.80
0	4.61	5.67	2.12
0	3.11	4.03	5.63
0	4.53	4.10	5.52
0	3.24	4.64	5.12
0	5.31	4.26	3.44
0	5.38	4.53	3.25
0	3.63	3.15	3.51
0	3.07	2.78	3.92
0	4.20	3.39	5.31
0	4.67	4.21	5.21
0	5.48	4.57	4.34
0	4.55	2.42	3.22
0	3.22	3.98	4.22
0	4.83	4.14	3.82

Table 8E (Continued)

Day	Shrimp weight (g)		
	Control	**Treatment 1**	**Treatment 2**
0	3.36	4.25	4.74
0	4.34	5.40	4.00
0	4.24	4.30	5.18
0	3.03	3.54	5.27
0	2.94	5.33	3.91
0	3.99	5.23	4.57
0	4.15	4.73	3.34
0	4.74	4.11	4.13
0	4.17	4.30	6.09
0	4.60	5.31	6.34
0	3.37	6.03	4.27
0	3.59	6.16	3.71
0	5.50	4.33	5.37
0	4.53	3.47	4.53
0	3.86	4.87	4.53
0	5.28	5.45	4.96
0	3.77	5.91	4.93
0	5.20	4.42	4.19
0	5.34	4.02	5.23
0	4.31	5.01	4.08
0	5.60	4.56	3.09
15	4.48	3.75	5.35
15	4.38	5.00	5.80
15	5.23	4.85	4.98
15	6.68	4.88	5.98
15	5.45	5.63	4.58
15	4.68	5.63	5.33
15	4.98	5.48	5.53
15	6.23	6.15	5.25
15	4.98	6.48	4.63
15	4.93	3.68	5.18
15	4.43	4.13	4.33
15	4.88	5.95	4.73
15	4.53	3.93	4.78
15	6.33	5.83	4.70
15	2.45	3.85	4.23
30	4.93	5.05	5.63
30	3.45	6.30	7.83
30	5.75	4.93	5.25
30	4.58	5.60	5.60
30	4.93	5.08	6.40
30	4.50	6.93	5.55
30	6.63	5.50	5.18
30	5.03	4.75	3.98
30	6.23	5.83	5.75
30	5.90	5.18	5.50
30	4.75	4.73	4.50
30	3.95	5.38	4.60
30	5.63	4.05	5.58
30	5.90	3.78	5.38
30	4.70	4.98	4.63
30	4.83	2.98	4.35

Table 8E (Continued)

Day	Shrimp weight (g)		
	Control	**Treatment 1**	**Treatment 2**
30	3.25	3.25	
45	5.13	6.85	6.45
45	6.05	4.45	5.63
45	3.38	6.00	5.75
45	6.08	5.65	6.88
45	4.95	6.18	4.60
45	7.05	6.83	5.20
45	5.03	5.58	8.33
45	5.35	8.25	6.90
45	4.95	8.03	8.10
45	7.58	5.58	6.75
45	3.80	4.45	6.03
45	4.03	6.08	6.65
45	5.03	3.08	6.28
45	4.10	4.88	5.38
45	5.63	4.10	4.43
45	-	-	7.20
45	-	-	4.80
45	-	-	4.53
45	-	-	7.13
45	-	-	6.18
45	-	-	7.38
45	-	-	8.10
45	-	-	5.78
45	-	-	4.25
45	-	-	6.60
45	-	-	5.25
45	-	-	4.45
45	-	-	6.45
45	-	-	6.05
45	-	-	4.75
45	-	-	4.85
57	6.03	8.43	7.83
57	5.38	6.65	7.43
57	3.50	7.43	7.55
57	8.53	8.98	6.83
57	6.15	5.93	8.48
57	6.40	5.70	7.88
57	5.55	5.55	7.33
57	4.88	5.75	7.63
57	4.95	5.20	6.75
57	6.15	5.05	7.23
57	5.50	6.35	5.28
57	3.45	4.93	6.35
57	5.93	8.18	8.70
57	6.08	6.65	6.13
57	6.30	6.10	5.50
57	5.60	6.78	5.93
57	4.35	7.35	6.55
57	8.38	7.23	5.05
57	6.28	6.30	5.85
57	4.50	6.80	9.25

Table 8E (Continued)

Day	Shrimp weight (g)		
	Control	**Treatment 1**	**Treatment 2**
57	4.58	6.08	9.50
57	5.10	7.70	9.75
57	6.15	5.25	7.98
57	-	7.00	6.60
57	-	6.70	9.35
57	-	5.70	6.75
57	-	6.88	7.78
57	-	4.98	9.23
57	-	5.30	6.63
57	-	6.98	8.93
57	-	6.75	6.90
57	-	6.55	5.18
57	-	6.30	7.98
57	-	5.25	5.78
57	-	-	5.33
57	-	-	6.45
57	-	-	5.95
57	-	-	5.70
57	-	-	7.10
57	-	-	6.73
57	-	-	6.15
57	-	-	5.83
57	-	-	5.80
57	-	-	6.35
57	-	-	8.18
57	-	-	8.30
57	-	-	5.53
57	-	-	7.83
57	-	-	5.15

BIOGRAPHY

Miss. Benjamas Chuntapa was born on January 8, 1971 in Khonkaen Province. She graduated with a Bachelor degree in Fisheries from the Department of Fisheries, Faculty of Agriculture, Khonkaen University, Khonkaen Province in 1993. After graduation, she continue to M.S. program at the Department of Marine Science, Chulalongkorn University in 1993. After receiving M.Sc. degree, she was working in the Environment Division, Department of Mineral Resource, Ministry of Industry. In 1998, she started the Ph.D program of study at the Marine Science at the Department of Marine Science, Faculty of Science, Chulalongkorn University.

During her M.S. study at the Department of Marine Science, Chulalongkorn University, she was under supervision of Professor Dr. Somkiat Piyatiratitivorakul and received scholarship from Thailand Research Fund (TRF) senior scholar research program which belonged to Professor Dr. Piamsak Menasveta.

During her Ph.D, she had an opportunity to work at Laboratory of Aquaculture and Artemia Reference Center, University of Gent, Belgium under financial supports from TRF Kanchanapisek Ph.D. scholarship.

She also had an opportunity to present a research paper on the improvement of water quality in shrimp pond by integrating culture of shrimp with the microalga, *S. platensis* at the Fourth Asia-Pacific Marine Biotechnology Conference, University of Hawaii at Monoa, USA.

After graduation, she will serve as an instructor at the Department of Science, Faculty of Science, Rajabhat Institute Rajanagarindra, Chachoengsao.

Research Publications:

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