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Integrated Aquaculture System of Fish, Microalga, Zooplankton and Aquatic plants

Mr. Puchong Sri-uam

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By	Mr. Puchong Sri-uam
Field of Study	Chemical Engineering
Thesis Advisor	Associate Professor Prasert Pavasant, Ph.D.
Thesis Co-Advisor	Sorawit Powtongsook, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Engineering
(Professor Bundhit Eua-arporn, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Muenduen Phisalaphong, Ph.D.)
.....Thesis Advisor
(Associate Professor Prasert Pavasant, Ph.D.)
.....Thesis Co-Advisor
(Sorawit Powtongsook, Ph.D.)
.....Examiner
(Associate Professor Artiwan Shotipruk, Ph.D.)
.....Examiner
(Assistant Professor Kasidit Nootong, Ph.D.)
.....External Examiner
(Wipawan Siangdung, Ph.D.)

ภุชงค์ ศรีอ่วม : ระบบบูรณาการการเพาะเลี้ยงสัตว์น้ำ ของปลา จุลสาหร่าย แพลงก์ตอนสัตว์ และพืชน้ำ (Integrated Aquaculture System of Fish, Microalga, Zooplankton and Aquatic plants) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ประเสริฐ ภวสันต์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.สรวิศ เผ่าทองศุข, 105 หน้า.

งานวิจัยนี้ได้นำหลักวิศวกรรมมาใช้ในการออกแบบระบบหมุนเวียนน้ำสำหรับการเพาะเลี้ยงสัตว์น้ำแบบปิด ด้วยการใช้ของเสียที่เกิดขึ้นจากหน่วยหนึ่งเป็นอาหารให้กับสิ่งมีชีวิตในหน่วยอื่นๆ ที่เชื่อมต่อกันเป็นระบบนิเวศที่เรียกว่า Integrated Multi-Trophic Recirculating Aquaculture System (IMRAS) งานวิจัยนี้แบ่งการทดลองเป็น 2 ส่วนหลัก คือ ส่วนที่ 1 (การปรับการสะสมสารอาหารในจุลสาหร่าย) โดยมีวัตถุประสงค์คือ ศึกษาผลของปัจจัยทางสิ่งแวดล้อม (ความเข้มแสง อุณหภูมิ และอัตราการให้อากาศ) ต่อการสะสมสารชีวเคมี (ไขมัน โปรตีน คาร์โบไฮเดรต) และการผลิตลูทีนในจุลสาหร่ายสีเขียว *Chlorella* sp. จุลสาหร่ายถูกเพาะเลี้ยงในถังปฏิกรณ์ชีวภาพเชิงแสงแบบคอลัมน์ ปริมาตร 2 ลิตร จำนวน 6 ขวด ภายใต้ตู้ที่สามารถควบคุมอุณหภูมิ ความเข้มแสง และอัตราการให้อากาศ ที่อยู่ในช่วง 30-40 องศาเซลเซียส (± 0.5องศาเซลเซียส) 10-30 กิโลลักซ์ (± 0.1 กิโลลักซ์) และ 0.5-1.5 เซนติเมตรต่อวินาที (± 0.05 เซนติเมตรต่อวินาที) ตามลำดับ ซึ่งผลการศึกษาพบว่าองค์ประกอบสารชีวเคมีในจุลสาหร่ายสามารถปรับได้ด้วยการควบคุมสภาวะการเพาะเลี้ยง โดยที่อัตราการผลิตไขมัน โปรตีนและลูทีนมากที่สุดที่สภาวะ อุณหภูมิ 35 องศาเซลเซียส ความเข้มแสง 10 กิโลลักซ์ และอัตราการให้อากาศ 1 เซนติเมตรต่อวินาที ในขณะที่ อัตราการผลิตคาร์โบไฮเดรตมากสุดที่สภาวะอุณหภูมิ 30 องศาเซลเซียส ความเข้มแสง 30 กิโลลักซ์ และอัตราการให้อากาศ 0.5 เซนติเมตรต่อวินาที ตามลำดับ

การทดลองส่วนที่ 2 เป็นการนำจุลสาหร่าย *Chlorella* sp. และพืชน้ำ มาใช้บำบัดน้ำเสียที่เกิดจากกระบวนการเลี้ยงปลา โดยทำการเพาะเลี้ยง *Chlorella* sp. แพลงก์ตอนสัตว์ (*Moina macrocopa*) และพืชน้ำร่วมกับการเลี้ยงปลานิล (*Oreochromis niloticus*) ที่ความหนาแน่น 20, 25, 50 ตัวต่อลูกบาศก์เมตร ที่สภาวะกลางแจ้ง ในระบบ IMRAS ผลการศึกษาแสดงให้เห็นว่า การพึ่งพาอาศัยกันที่เหมาะสมระหว่างสิ่งมีชีวิตหลากหลายสายพันธุ์ สามารถควบคุมระดับความเข้มข้นของแอมโมเนีย ไนไตรท์ ไนเตรต และฟอสเฟส ในระบบให้ต่ำกว่าระดับที่เป็นอันตรายต่อการเพาะเลี้ยงปลานิลตลอดช่วงเวลาการเพาะเลี้ยงได้ โดยที่อัตราการผลิตปลานิลโดยน้ำหนักสูงสุด คือ 11±1 กิโลกรัมต่อลูกบาศก์เมตร (ที่ความหนาแน่นปลานิลเริ่มต้น 50 ตัวต่อลูกบาศก์เมตร) พืชน้ำสามารถดูดซึมปริมาณไนโตรเจนและฟอสฟอรัสได้ประสิทธิภาพสูงสุด 9.52 และ 11.4 เปอร์เซ็นต์ โดยที่อัตราการดูดซึมไนโตรเจนและฟอสฟอรัสของพืชน้ำมีความแตกต่างกันในแต่ละสายพันธุ์โดยเรียงลำดับจากมากสุดไปน้อยสุด ดังนี้ สาหร่ายเดนซ่า สาหร่ายพุงชะโด เทปตรง เทปเกลียว และ ดาวกระจาย ตามลำดับ ซึ่งสารประกอบไนโตรเจนที่เหลืออยู่ในระบบประมาณ 39.67% ถูกเปลี่ยนรูปไปเป็นก๊าซไนโตรเจนโดยผ่านกระบวนการดีไนตริฟิเคชั่น ในขณะที่ฟอสฟอรัสที่เหลืออยู่ในระบบประมาณ 54.46% จะตกตะกอนสู่ดินภายในถังบำบัด

ภาควิชา วิศวกรรมเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา ว□ศวกรรมเคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา 2557	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5271819021 : MAJOR CHEMICAL ENGINEERING
KEYWORDS: CHLORELLA SP. / BIOCHEMICAL COMPOSITION / AQUATIC PLANT / MULTI-TROPHIC / RECIRCULATING AQUACULTURE SYSTEM

PUCHONG SRI-UAM: Integrated Aquaculture System of Fish, Microalga, Zooplankton and Aquatic plants. ADVISOR: ASSOC. PROF. PRASERT PAVASANT, Ph.D., CO-ADVISOR: SORAWIT POWTONGSOOK, Ph.D., 105 pp.

This work was designed to give engineering detail for the design of a recirculating aquaculture system where wastes from one part are being consumed as feed for other organisms in the whole concocted ecosystem (Integrated Multi-Trophic Recirculating Aquaculture System, IMRAS). The experiments were separated into 2 main parts. Part I (Algal nutrition adjustment) aimed to study the effect of several environmental parameters (light intensity, temperature, and aeration rate) on the accumulation of nutritional components and lutein production in a green microalgae *Chlorella* sp. It was proven in this work that the biochemical composition of *Chlorella* sp. could be manipulated through the control of environmental conditions during the cultivation. Six simple 2L bubble column photobioreactors installed in a well controlled culture chamber were employed as a model system where temperature, light intensity, and aeration rate (u_{sg}) could be controlled in the range from 30-40ºC (± 0.5ºC), 10-30 kLux (± 0.1 kLux), and 0.5-1.5 cm/s (± 0.05 cm/s), respectively. Lipid and protein productivity were the most abundant at 35°C, 10 kLux and 1 cm/s, whereas carbohydrate productivity was found to be maximized at 30°C, 30 kLux and 0.5 cm/s. In addition, *Chlorella* sp. could also generate strong antioxidizing agents like lutein which was found to be mostly produced at 35°C, 10 kLux and 1 cm/s.

Part II (Outdoor cultivation) aimed to study outdoor cultivation of integrated multi-trophic recirculating aquaculture system, where *Chlorella* sp. and aquatic plants tanks acted as a nitrogen conversion unit. In this work, three densities of the sex-reversed male Nile tilapia, *Oreochromis niloticus* (20, 25, 50 fish/m^3) were cultivated in IMRAS that involved the ecological relationship between several living organisms, i.e. phytoplankton, zooplankton, and aquatic plants. The results indicated that, by providing proper interdependency between various species of living organisms, the concentrations of ammonia, nitrite, nitrate, and phosphate in the system were controlled below dangerous level for Nile tilapia throughout the cultivation period. The highest wet weight productivity of Nile tilapia of 11±1 kg/m^3 was achieved at the initial fish density of 50 fish/m^3. The aquatic plants in the treatment tank could effectively uptake the unwanted nitrogen (N) and phosphorus (P) compounds with the highest removal efficiencies of 9.52 and 11.4%, respectively. The uptake rates of nitrogen and phosphorus by aquatic plants could be ordered from high to low as: *Egeria densa* > *Ceratophyllum demersum* > *Vallisneria spiralis* and *Vallisneria Americana* > *Hygrophila difformis*. The remaining N (39.67%) was further degraded through denitrification process whereas the remaining P (54.46%) could well precipitate in the soil sediment in the treatment tank.

Department:	Chemical Engineering	Student's Signature	.....
Field of Study:	Chemical Engineering	Advisor's Signature	.....
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INTRODUCTION

1.1 Motivations

Recirculating aquaculture system and Multi-trophic aquaculture

Typical aquaculture systems are mono-culture where the target aquaculture species such as fish or shrimp is cultivated in a batch mode. The system has to be large enough that the left over feed and wastes from the culture are being naturally treated with several types of microorganisms inhabited within the system. However, it is quite common to have a high density culture where the system has to be fed with a large quantity of feed and in certain cases with extra aeration. In this case, problems always arise when the wastes could not be adequately treated resulting in an unsuitable living conditions for the culture. This can lead to stress which negatively affects the growth and the productivity of the system.

One attempt to deal with this problem is to have a recirculating system where the unwanted wastes are taken out of the culture tank and being treated very effectively elsewhere. Examples include the nitrification/denitrification units which could be designed such that the rate of nitrogen removal matches that of nitrogen generation due to growth. In multi-trophic systems, on the other hand, such wastes will be used as a feed for other organisms, e.g. aquatic plants, simulating the symbiosis relationship in natural ecosystems.

The design of this multi-trophic aquaculture is quite important as this will affect the economics of the aquaculture. A well selected food-chain-like organisms enables the farmers to generate more incomes from the by-products that can be harvested from the system. In this work, this multi-trophic recirculating aquaculture system was based on Nile tilapia as the major species. Nitrogen excreted from the fish is used as a feed for microalgae which in this case is *Chlorella* sp. This algal species could grow reasonably well in tropical climate and therefore could be cultured with minimal maintenance. The biomass of *Chlorella* sp. is fed to *Moina macrocopa* tanks

which can be more easily harvested when compared to *Chlorella* sp. in which its small size leads to harvesting difficulties. The remaining nitrogen waste is used to grow aquatic plants which can also be harvested, where the clean water is recirculated back to the fish tank. With this configuration, the system will more effectively utilize the feed and the farmers will earn benefits from having a variety of products apart from the major aquacultural species.

This work focuses on the different components in this multi-trophic culture and describes some target organisms in more detail as shown below.

***Chlorella* sp culture and its biochemical composition**

Chlorella sp. has been widely known and cultured for a number of applications including food source for fish hatchery (Ashraf et al., 2010, Duray et al., 1997, Hirayama et al., 1989), animal food additives (Gouveia et al., 1996), human food supplementary (Chu, 2011, Kanno, 2005, Park & Kim, 2012, Yang & Huang, 2012), a biodiesel feedstock (Phukan et al., 2011), and even a wastewater treatment agent. This microorganism can grow competently under non-strictly specified conditions. In other words, it can grow under temperature range of as wide as 4-35ºC, light intensity of 1,000-70,000 Lux, and aeration rate of 0-6 L/min (Belkoura et al., 1997, Phukan et al., 2011, Ramazanov & Ramazanov, 2006, Teoh et al., 2004). This has made it one of the most versatile strains in algal culture industry. *Chlorella* can also be grown under different growth conditions to enable the accumulation of various specific components. For example, for a food source for fish hatchery or food additives, the alga is normally cultured under conditions urging the cell to accumulate high amount of protein (Seyfabadi et al., 2011, Teoh et al., 2004). As human supplementary food, the cell must be cultured such that carbohydrate could be accumulated as much as practicable (Lv et al., 2010). On the other hand, if the product is biodiesel feedstock, the cell is stressed to produce the maximal amount of lipid (Yeh et al., 2010).

In certain applications, high value products, e.g. antioxidants, might be needed and the alga must be grown in the right environment to be able to store such compounds (Guedes et al., 2011). Commercial lutein is produced from marigold but

this suffers some disadvantages particularly long cultivation time and large area requirement. In this regard, microalgae can be an alternative source with comparable or even higher lutein productivity. For instance, lutein productivity from outdoors cultivation of *Muriellopsis* sp. was 180 mg/m^2/d (Del Campo et al., 2001), approx. 11 times higher than that from marigold (Prommuak, 2012). Several microalgae can effectively produce lutein such as *Scenedesmus almeriensis* (Sánchez et al., 2008b, Sánchez et al., 2008a), *Chlorella protothecoides* (Wei et al., 2008), *Chlorella zofingiensis*, *Chlorococcum citriforme* and *Neospongiococcus gelatinosum* (Del Campo et al., 2000).

Nitrogen removal in recirculating aquaculture system

Recirculating aquaculture system (RAS) is an integrated close cultivation system where the circulation between the cultivation and the treatment tanks helps maintain the quality of water. This clean water allows a better control of disease (Bahnasawy et al., 2009) and promotes a better growth of the aquatic animals which enhances the productivity of the system. In addition, the treatment tank can also act like a holding basin when the cultivation tank needs to be emptied for maintenance. This ability to collect clean water eliminates the need of water from the external irrigation system and prevents unnecessary contamination from external sources. In RAS, the wastewater from aquatic animal culture, which contains mostly nitrogen and phosphorus compounds, is not only treated by the typical nitrification and denitrification processes taken place in the biological filters (Menasveta et al., 2001, Silapakul et al., 2005, Uemoto & Morita, 2010, Wei et al., 2010, Zhang et al., 2010), but also by the activities induced in the treatment tank such as the uptake of vegetable/ornamental plants (Lewis et al., 1978, Naegel, 1977, Seawright et al., 1998, Thanakitpairin et al., 2014, Trang & Brix, 2014) or aquatic plants. As the role of *Chlorella* in wastewater treatment, due to the need of nitrogen for its growth, *Chlorella* could be applied for aquaculture wastewater treatment since nitrogen presented in nitrate form is normally found in its excrement (Zheng & Wang, 2010). Furthermore, in Thailand, most cultivation of *Chlorella* is used as live feed for zooplankton (e.g. water fleas and rotifers) which will thereafter be used as

aquatic animal feed. Therefore, the developments of *Chlorella* cultivation for aquaculture are of interest.

Regarding to aquaculture, Nile tilapia, *Oreochromis niloticus*, is one of the most important cultured fish species due to its several advantages over others, e.g. fast growth, adaptability to a wide range of environmental conditions, ready acceptance of artificial feed, and ease of reproduction (Pechsiri & Yakupitiyage, 2005). To achieve high yield of Nile tilapia, the culture is commonly carried out in the intensive systems such as cages. The cage culture is advantageous over a common earth pond culture as the feeding is easy and effective, resulting in a high growth rate. Also the cage allows the easy migration management and harvest with minimum investment. However, there are disadvantages from the cage culture of Nile tilapia. For example, the uncontrollable water diseases could affect the whole cage, the interference from wild fish could contend over for food or probably kill the fish, the density of cage cultures could pose some serious concerns on natural water source quality as the exchange of water from natural water sources could be unavoidable, and fish excrement and food scraps might be piled up at the bottom of the pond (particularly still water), resulting in sewage. In addition, it requires more time and labors than other culture systems especially for monitoring and controlling the system. To elucidate these problems, the model of the open culture, by which the culturing water is treated and recirculated within the farm, would be revolved to the closed recirculating system.

In the developed closed system, there is still problematic quality of water since ammonia as the main constitute of fish excrement normally turns to toxic nitrite (NO_2) and nitrate (NO_3) via nitrification process. In general, ammonia and nitrite are both seriously toxic to aquatic animals but nitrate is less toxic than the first two. However, the excess nitrate can lead to algae bloom which later on causes the water pollution. Normally, the elimination of nitrate from the closed aquaculture pond can be carried out by changing NO_3 to N_2 through denitrification reaction (see Chapter II). However, the economics of such denitrification process is still doubtful due to the fact that the reaction must be carried out only in the specific low oxygen, anoxic condition (DO<1.5 mg/L). Moreover, methanol or other organic chemical addition is needed for the growth of denitrifying bacteria, and most of all, N_2, the final product of the

reaction is considered no economic value. Luckily, nitrate is an important nitrogen source for several aquatic plant species, such as *Egeria densa* (Densa) and *Ceratophyllum demersum* L. (Common coontailor) etc., used for fish tank decoration, and microalgae such as *Chlorella vulgaris*, *Scenedesmus* sp., etc. (Touchette & Burkholder, 2000). Hence, the elimination of nitrogen compound from aquaculture effluent in RAS by turning it into aquatic plants and microalgae should be considered as a potential answer to this water quality problem. By providing a proper balance between these various species, this RAS could shares the common important concept with the integrated multi-trophic aquaculture system (IMTA) where the synergistic relationship between the living organisms helps promote the sustainability and the economics of the whole system.

Proposition

The purpose of this work can be divided into two parts. Firstly, the optimal conditions of *Chlorella* sp. cultivation for the biochemical production were studies. Secondly, an outdoor of Nile tilapia closed culture system was investigated where the fish tank medium was circulated as a nitrogen and phosphorous source for microalgae *Chlorella* sp. and aquatic plants before returning to the fish tank. Not only does this research help enhance the water quality, leading to a minimum requirement for water exchange with other natural sources; it does also help the fish farmers to earn more revenue from the trade of these aquatic plants species.

1.2 Objectives

The objectives of this work aimed to:

1) Study the effect of several environmental parameters (light intensity, temperature, and aeration rate) on the accumulation of nutritional components in a green microalgae *Chlorella* sp.

2) Design and investigate the closed loop outdoor fish cultivation system where *Chlorella* sp. and aquatic plants tank takes part as a nitrogen and phosphorous conversion unit.

1.3 Working Scopes

The scopes of this work were separated into 2 parts. Part 1: "Algal nutrition adjustment" was the study of the effect of several environmental parameters on the accumulation of nutritional components. Part 2: "Integrated multi-trophic recirculating aquaculture system (IMRAS) for Nile tilapia" was the study of the closed loop outdoor aquaculture system where wastes from one part were consumed as feed for other organisms in the whole concocted ecosystem. Figure 1.1 summarizes the concept of this work where the scopes of each part are described as follows:

Part 1 (Algal nutrition adjustment):

1) Temperature: 30, 35, and 40 ºC

2) Light intensity: 10, 15, 20 and 30 kLux

3) Aeration rate (without CO_2): 0.5, 1 and 1.5 cm/s (1.7, 3.4 and 5.1 vvm)

4) Photobioreactor: bubble column (2 L clear bottle)

5) Culture medium: modified M4N

Part 2 (Integraed multi-trophic recirculating aquaculture system for Nile tilapia):

1) Amount of Nile tilapia: 20, 25, and 50 fish/m^3

2) Phytoplankton (*Chlorella* sp.) and zooplankton (*Moina macrocopa*)

3) Aquatic plants: *Egeria densa*, *Ceratophyllum demersum*, *Hygrophila difformis*, *Vallisneria spiralis*, and *Vallisneria Americana*

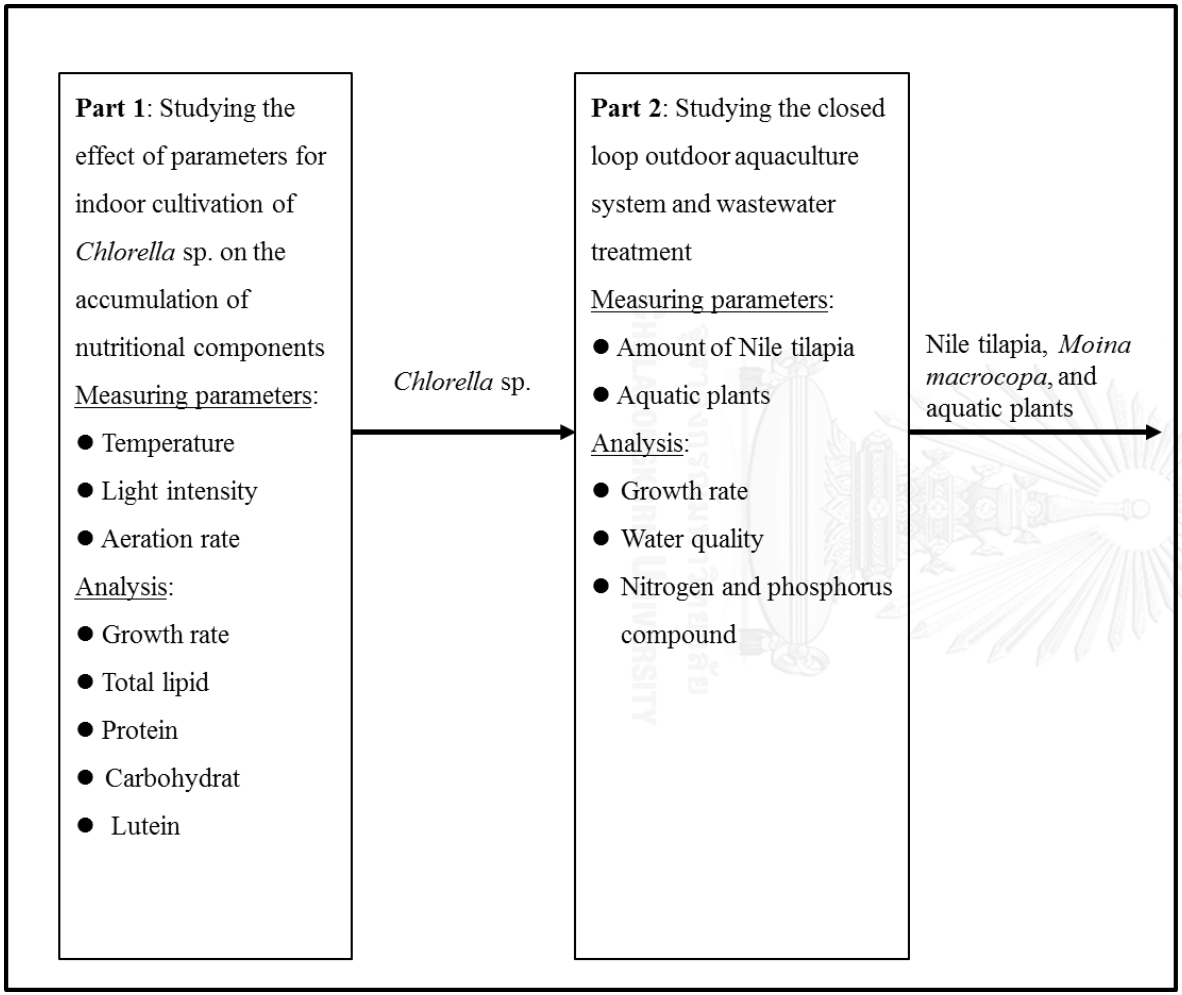

Figure 1.1 Conceptual design for the experiment

CHAPTER II
BACKGROUNDS AND LITERATURE REVIEW

2.1 Phytoplankton: *Chlorella* species

Chlorella sp. is a green microalga without flagella. The shape of cell is spherical with diameter of 2 to 10 μm. *Chlorella* sp. is also important food for herbivorous aquatic animal larvae such as fish, shrimp and zooplankton like water flea, copepod, rotifer, etc (Al-Shamsi *et al.*, 2006, Garcia *et al.*, 2003, Rajendiran & Subramanian, 2007). Exploitation of *Chlorella* sp. for bioenergy generation especially biodiesel (Chisti, 2007, Li et al., 2007, Miao & Wu, 2004, Miao et al., 2004, Scragg et al., 2003, Xu et al., 2006) , wastewater treatment (Chu et al., 2009, Min et al., 2011), carotenoids production (Del Campo et al., 2004, Fernández-Sevilla et al., 2010) and CO_2 mitigation, by which CO_2 is captured and sequestered (Huntley & Redalje, 2007).

2.1.1 *Chlorella* cultivation

The most important parameters regulating *Chlorella* growth are nutrient, light intensity, temperature, pH, and aeration (Lavens et al., 1996). Details are delineated below.

- Nutrients

Cell density in the medium cultures is generally higher than those found in nature. Microalgal cultures must be enriched with nutrients to make up for the deficiencies in the water. Macronutrients include nitrate, phosphate and silicate. Micronutrients consist of several trace metals and the vitamins thiamin (B1), cyanocobalamin (B12) and occasionally biotin. The enrichment media that have been used widely for *Chlorella* cultivation with various formulas such as the modified M4N (Kumoro & Susanto, 2013, Sung et al., 1999), N-8 (Vonshak, 1986), M-8 (Mandalam & Palsson, 1998), BG-11 medium (Rippka & Herdman, 1992) etc.

- Light intensity

Light is the source of energy to drive photosynthesis reaction where light intensity and photoperiod need to be considered. The culture has been direct sunlight or high artificial light intensity cause photoinhibition. The requirements vary with the culture depth and the density of the algal culture where higher depths and cell concentrations the light intensity should increase to penetrate through the culture.

- Temperature

Temperature is an important for cell growth and biochemical composition. The optimal temperature of *Chlorella* is various with species i.e. *Chlorella kessleri, Chlorella Pyrenoidosa* - Sorokin strain and *Chlorella* sp. R-06/2 cultures are 34-36°C (Kessler, 1985), 37-39°C (Hannan et al., 1963) and 42 °C (Gacheva & Pilarski, 2008), respectively.

- Aeration/mixing

Mixing is necessary to prevent sedimentation of the microalgae, to ensure that all cells are equally exposed to the light and nutrients, avoid thermal stratification and improve mass transfer. The mixing is performed using stirring, aerating and paddle wheels.

2.1.2 Lipid, protein and carbohydrate accumulation behavior of *Chlorella* microalgae

The cultivation of *Chlorella* has been widely studied. Many studies reported that temperature and light intensity normally applied for the cultivation of microalgae are in the range of 22-35^{o}C and 1,200-66,600 Lux, respectively. This depends on the diversity of species as described in Table 2.1. Culture conditions of *Chlorella* sp. have been found to give significant effects on its behavior in accumulating nutritional components such as protein, carbohydrate and lipid. Seyfabadi et al. (2011) revealed

that at 25^{o}C with the light-dark cycle of 16:8, protein accumulation in *Chlorella vulgaris* was found to increase from 36±2.2 to 43±3 and 46±3.7 (%) with the increase of light intensity from 2,775 to 4,625 and 7,400 Lux, respectively. Table 2.1 shows that *Chlorella* sp. is able to accumulate lipid, protein and carbohydrate from the range of 13-40, 10-78 and 6-67% by its dry weight, respectively. *Chlorella* UMACC 237 was reported to have the highest protein accumulation at 9^{o}C and light intensity of 3,108 Lux. (Teoh et al., 2004). *Chlorella* vulgaris ESP-31 was reported to have the highest lipid accumulation at 25^{o}C and light intensity of 9 W/m^{2} (Yeh et al., 2010), while under the cultivation temperature and light intensity of 25^{o}C and 4,440 lux, the cell could accumulate the highest amount of carbohydrate (Lv et al., 2010).

Other microalgae that have been studied for the accumulation of lipid, protein and carbohydrate regarding their cultivation conditions are listed in Table 2.2. These are such as *Nannochloropsis* sp. (Sayegh & Montagnes, 2011), *Isochrysis galbana* (Sánchez et al., 2000, Sayegh & Montagnes, 2011), *Choricystis minor* (Mazzuca Sobczuk & Chisti, 2010), *Spilurina platensis* (Yilmaz et al., 2010), *Chaetoceros calcitrans f. pumilus* (Gireesh et al., 2008), *Amphora* sp. (De la Pena, 2007), *Dunaliella tertiolecta* (Takagi et al., 2006), *Nannochloropsis oculata* CS-179 and *Isochrysis* sp. CS-177 (Renaud et al., 1991).

It can be concluded from Tables 2.1 and 2.2 that the cultivation must be operated under specific conditions to obtain microalga with the required properties. For example, to increase lipid accumulation, the cultivation should be carried out under the condition of nitrogen starvation (Ramazanov & Ramazanov, 2006), light reduction (Belkoura et al., 1997) or temperature elevation (Sayegh & Montagnes, 2011). On the other hand, for the increase of protein accumulation, microalgae should be cultured in extra light intensity with longer duration of light exposure (Seyfabadi et al., 2011) at mild temperature (Brown & Jeffrey, 1992, Teoh et al., 2004) or sometimes at elevated temperature (Belkoura et al., 1997, Gireesh et al., 2008, Sayegh & Montagnes, 2011). In case where a high accumulation of carbohydrate was needed, the algae were subject to an extending cultivation duration (Lv et al., 2010), starving from nitrogen (Ramazanov & Ramazanov, 2006), increasing light intensity (Belkoura et al., 1997) or cultivation temperature (Sayegh & Montagnes, 2011).

2.2 Zooplankton: *Moina* spp.

Moina spp. or "water fleas" is small organism which sizes are in the range from 0.4 to 1.8 millimeters. The numbers of *Moina* become abundant in transient water where the optimal temperature is 24-31°C (Rottmann et al., 2004). *Moina* reproduce both a sexual and asexual phase depend on environmental condition. Normally, all females reproduce asexually where the life span is between 4 to 6 days. Under optimum conditions, *Moina* propagate 1 to 5 times or average of 3 times for 19 to 23. On the other hand, sexual reproduction could be take place under unfavorable conditions where resting eggs (ephippia) are produced.

Moina consume phytoplankton such as *Chlorella, Chlamydomonas, Scenedesmus* and yeast are classified as a type of *Daphnia*. With rapid multiplication, fresh *Moina* are popularly cultured as food for many fancy fish as well as economic larval fish e.g. Sleepy goby and Tilipia due to its high protein content (approximately 50% w/w). Therefore, likewise food chain, *Chlorella* is often simultaneously loaded into nursery ponds as food for *Moina*. Moreover, the addition of *Chlorella* into fish ponds can help protect the larvae from sunlight and get rid of waste, especially dissolving ammonia and nitrate. This can also improve water quality. However, in Thailand, most of *Moina* cultivations are carried out by typical systems where the microalgae, as food for *Moina*, are firstly grown individually in a 50 square meter open pond. With proper inoculum, *Moina* can reach their optimal cell concentration in approximately 4-7 days. The *Moina* are harvested when the dark "green medium" turns into slightly greenish color. Hence, the green microalga cultivation is considered a very important first step for *Moina* cultivation. In the other word, the cultivation of *Moina* would not be successful without an efficient microalgal cultivation that can produce the green algae with adequate amount.

Moina cultures can be combined with phytoplankton or separate culture unit. The combined cultures are a simple method, but the production is lower than the separate culture. For separate culture, the microalgae medium flow into *Moina* culture tank and then *Moina* is separated using plankton net (Figure 2.1). The advantages of this method are less contamination, easy to control, and more consistent yield production, however, the disadvantage is to use more space.

2.3 Aquatic plants

Aquatic plants live in aquatic environments that are found both of saltwater and freshwater. The factor regulating aquatic plants growth is the depth and duration of flooding including nutrients, and salinity. Some aquatic plants are used by humans as a food source such as water caltrop (*Trapa natans*), Indian lotus (*Nelumbo nucifera*), Chinese water chestnut (*Eleocharis dulcis*) and water spinach (*Ipomoea aquatica*) etc. Aquatic plant uses sunlight as energy to build biomass, while dissolved inorganic nutrients are removed from the water. The aquatic plant can utilize pollutant nutrients as their food and energy source, clean the water, and be harvested as commercial crops with very little added cost to the producer. Recycling of waste nutrients by aquatic plants and filter-feeders is the most economical way to improve aquaculture sustainability.

2.4 Nile tilapia (*Oreochromis niloticus*)

Nile tilapia is a tropical freshwater fish species (*Cichlidae* family) originated in the African continent. Shape of Nile tilapia likes Mozambique tilapia. The characteristics of Nile tilapia are the consistent upper and lower lips, with scales fourth straight rows on cheeks. A diagonal stripes across the body about 9-10 bar. Nile tilapia had a habit of living together in groups along rivers, wetlands and lakes. They can live in both shallow freshwater and brackish water. The optimum environmental temperature is between 30 and 36 °C whereas fish become lethal when the temperature is lower 11°C or upper 40°C. Nile tilapia can consume all kinds of feed such as periphyton, algae, aquatic plant, duckweed, insect larvae and small animals that live in the pond. To make the fast growth rate of Nile tilapia, bran grits, soybean meal, peanut meal, and fish meal should be feed. Amount of each feeding should not over excess where weight of feed is around 5% of the total weight of the fish.

The aquaculture system is divided into three type i.e. open system, semi-closed system and closed system. The open system uses water from natural sources, or a

simple filtration system, after that the water is discharged to natural water reservoir without water treatment and the water is reused again. Semi-closed system, some of water is reused which the water will be treated before discharge to the natural water reservoir. The closed system is a system that wastewater from aquaculture activity does not be discharged to the outside and the water will be treated with various methods such as biological filter. The Nile tilapia can culture in several types of container such as ponds, tanks and cages.

2.5 Nitrification and denitrification

Nitrogen compounds may be biologically oxidized to nitrates, provided that proper aerobic environment is provided. Should the nitrified effluent be subjected to a period of anaerobiosis, the bacteria can utilize nitrate as electron acceptors. Under these conditions, nitrates and nitrites are reduced to nitrogen gas. This process leads to a reduction in the nitrogen content of the water in gaseous form.

All of the system designs proposed for nitrogen removal process are based on the sequential steps of the oxidation of nitrogenous material (Nitrification) and the reduction of nitrates (Denitrification), and these are delineated below.

2.5.1 Nitrification process

Nitrification is an aerobic, autotrophic oxidation of ammonia to nitrite and to nitrate by autotrophic organisms which derive their energy solely from these oxidations and not from the oxidation of reduced carbon compounds (Wallace & Nicholas, 1969). This type of microorganisms utilizes carbon dioxide, bicarbonate or carbonate as a carbon source for biosynthetic process and oxidation of reduced nitrogen compounds as an energy source (Strotmann & Windecker, 1997). The first oxidation step, e.g. the conversion of ammonium ions to nitrite is carried out mainly by *Nitrosomonas* species, although other genera, including *Nitrosococcus* and *Nitrosospira* may also complete the task. Some subgenera, *Nitrosolobus* and *Nitrosovibrio*, can also autotrophically oxidize ammonia (Watson et al., 1981). These nitrite-oxidizing bacteria oxidize ammonium to nitrite according to Equation (2.1).

$$\mathbf{NH_4^+ + 1.5O_2 \rightarrow\ NO_2^- + H_2O + 2H^+} \quad (2.1)$$

In the second step of the process, nitrite-oxidizing bacteria oxidize nitrite to nitrate according to Equation (2.2).

$$\mathbf{2NO_2^- + O_2 \rightarrow 2NO_3^-} \quad (2.2)$$

Nitrobactor is the most frequently identified genus associated with this second step, although other genera, including *Nitrospina*, *Nitrococcus*, and *Nitrospira* can also autotrophically oxidize nitrite (Watson et al., 1981).

The overall nitrification which is the combination of the above two stoichiometries is:

$$\mathbf{NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+} \quad (2.3)$$

The overall nitrification reaction indicates that the oxygen requirement for the oxidation of ammonia is 4.57 g O_2/g NH_4^+-N, which consists of 3.43 and 1.14 g O_2 for the oxidations of ammonium and nitrite, respectively (neglecting cell synthesis). Randall et al. (1991) proposed the reaction stoichiometry for the reaction with cell synthesis as:

$$\mathbf{NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.98NO_3^- + 0.021cell + 1.88H_2CO_3 + 1.041H_2O} \quad (2.4)$$

Where approximately 4.18 g O_2/g NH_4^+-N was consumed for the oxidation of ammonia in this case. Comparing the two equations above, it could then be concluded that the cell synthesis had very little effect on the overall oxygen requirement for the nitrification and could be neglected. Hence, oxygen requirement from Equation (2.3) is usually considered for the nitrification reaction.

2.5.2 Denitrification process

Denitrification is the dissimilatory reduction of NO_3^- or NO_2^- to N_2 gas. In other words, NO_3^- and NO_2^- are the electron acceptors used in energy generation metabolism. Denitrification is widespread among heterotrophic and autotrophic bacteria, many of which can shift between oxygen respiration and nitrogen

respiration. Denitrification is applied when a complete removal of nitrogen is required. The reduction steps of nitrate can be illustrated as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (2.5)$$

The biological process of denitrification involves the conversion of nitrate nitrogen to a gaseous nitrogen species, primarily nitrogen gas. As opposed to nitrification, a relatively broad range of bacteria can accomplish denitrification, e.g. Pseudomonas, Micrococcus, Achromobacter and Bacillus (EPA., 1975). Many bacteria can shift between using oxygen and nitrate (or nitrite) rapidly and without difficulty. Denitrification is achieved by contacting nitrified wastewater with biomass in the absence of oxygen. Practically, the reduction of nitrate is accelerated by adding biologically degradable organic material to anaerobic step. Several early investigators added raw sewage to the denitrification basin to speed up the reaction, but this has the limitation of adding unoxided nitrogen compounds and additional BOD to the final effluent. Most recent investigators have used methanol to accelerate the biological denitrification. Stoichiometries involved in the denitrification are:

First step of denitrification:

$$NO_3^- + \frac{1}{3}CH_3OH \rightarrow NO_2^- + \frac{1}{3}CO_2 + \frac{2}{3}H_2O \quad (2.6)$$

Second step of denitrificatin:

$$NO_2^- + \frac{1}{2}CH_3OH \rightarrow \frac{1}{2}N_2 + \frac{1}{2}CO_2 + \frac{1}{2}H_2O + OH^- \quad (2.7)$$

Overall denitrification reaction:

$$NO_3^- + \frac{5}{6}CH_3OH \rightarrow \frac{1}{2}N_2 + \frac{5}{6}CO_2 + \frac{7}{6}H_2O + OH^- \quad (2.8)$$

Based on the above stoichiometry, one mole of nitrate requires at least five-sixth mole of methanol for complete denitrification, or 1.9 mg of methanol is required for each mg of nitrate-nitrogen. If the effluent contains dissolved oxygen, then it must be removed before denitrification can occur. This biological reaction can be accomplished by the addition of methanol as follows:

$$O_2 + \frac{2}{3}CH_3OH \rightarrow \frac{2}{3}CO_2 + \frac{4}{3}H_2O \quad (2.9)$$

Each mg of dissolved oxygen (DO) requires 0.67 mg of methanol (CH_3OH) for its removal. Methanol must also be supplied to satisfy the requirements for bacterial growth. The quantity of methanol is about 1.3 times of the amounts given in the

stoichiometric equation (Eq. 2.8). From these considerations, the following formula may be used for estimating the total amount of methanol required:

$$C_m = 2.47NO_3\text{-}N + 1.49NO_2\text{-}N + 0.87DO_i \quad (2.10)$$

where

C_m = required methanol concentration (mg/L)

NO_3-N = initial nitrate-nitrogen concentration (mg/L)

NO_2-N = initial nitrite-nitrogen concentration (mg/L)

DO_i = initial dissolved oxygen concentration (mg/L)

The value of C_m calculated above is somewhat conservative in that it does not make any allowance to the residual BOD entering the denitrification step. A methanol to nitrate-nitrogen ratio of 3.0 was suggested as a design guideline (EPA., 1975).

2.6 The improvement of quality of farming water

Without replacement of fish farming water, the accumulation of inorganic nitrogen compounds e.g. ammonia, nitrite and nitrate can occur. The high level of these compounds, especially ammonia and nitrite can cause serious damage and death to fish. Accordingly, the concentration of these nitrogen compounds must always be monitored and controlled. To minimize the amount of the compounds, various methods based on biological filtration have been widely applied. Each method, e.g. rotating biological contactor (RBC), tickling filters, submerged filter or fixed bed filter, fluidized bed reactor, expandable media filters or bead filter, airlift reactor, membrane reactor, and floating immobilized carriers, provides a habitat for beneficial bacteria to grow and will later on convert harmful nitrogen compounds into beneficial nitrates. However, each technique has different advantages and drawbacks, as listed in Table 2.3. As a matter of fact, nitrate can accumulate in water from the nitrification activity. An excessive amount of nitrate in the system results in “Algae Bloom” causing harm and death to the fish. Hence, some part of water must be replaced with newly fresh medium. However, the discharge of water full with nitrate into natural water sources is lawfully prohibited. For this reason, the concentration of nitrate in farming water must be ensured to be under the terms of law prior to discharging into public water resources, and this is the main concept of the “closed water fish farming

system". The most popular nitrate conversion method is called "Denitrification" as described earlier in this chapter. Depending on the type of biological filters, nitrification and denitrification can be induced at various rates. For example, via nitrification, RBC biological filtration was reported to be capable of removing nitrogen with the rate of 0.05-0.06 gN/m^2/day while trickling filters could treat nitrogen with the rate of 0.012-0.92 gN/m^2/day. For submerge filters, nitrogen was removed with the rate of 0.06-1.67 gN/m^2.day while it occurred to be 1.3-4.33 gN/m^2/day in case airlift reactors were employed. For denitrification, nitrogen could be treated at the rate of 0.007-0.025 $mgNO_3$-N/L/min using a fixed film column while that of 0.02-2.77 $mgNO_3$-N/L/min was observed when floating immobilized filtration was carried out. More details of nitrogen treatments via nitrification and denitrification can be found in Tables 2.4 and 2.5, respectively. One significant advantage of removing nitrogen via nitrification and denitrification is that they both provide efficient treatment. However, these microbial mechanisms only convert nitrogen compounds to N_2 which has no economic value.

2.7 The recirculating aquaculture system (RAS) and Integrated Multi-Trophic Aquaculture System (IMTA)

Recirculating aquaculture systems (RAS) technology has progressively established over the past 30 years (Murray et al., 2014). It is generally used for broodstock managing, in hatcheries and for grow-out stage of fish and shrimp production. RAS was aimed to reduce water consumption (Verdegem et al., 2006), control culture conditions (temperature, pH, salinity), and manipulate waste streams (Piedrahita, 2003) by mechanical, biological, chemical filtration and other treatment procedure. High density aquatic animal can be cultured in RAS, as the management of hygiene and disease is better than traditional aquaculture practices (Tal et al., 2009). In addition, another important feature of the RAS was to reduce nitrogen and phosphorus nutrients concentration and sediment of aquaculture effluents (Jegatheesan et al., 2007, Little et al., 2008). Aquaculture in this form was relatively environmentally friendly. For this reason, RAS are widespread around the world, including Europe (Holan et al., 2014, Martins et al., 2010), North and South America

(Auffret et al., 2013, Gilles et al., 2014, Merino et al., 2009, Park et al., 2015), Africa (Ajiboye & Yakubu, 2010, Akinwole & Faturoti, 2007, Gilles et al., 2013), Australia (Cahill et al., 2010, Le et al., 2013) and Asia (Burut-Archanai *et al.*, 2013a, Kikuchi *et al.*, 2006, Liu *et al.*, 2014).

Although aquaculture in RAS can reduce the emissions of nitrogen, phosphorus nutrients and sediment from intensive aquaculture effluents, but the toxicity of nitrate is still accumulated in the culture system. Schram et al. (2014) and van Bussel et al. (2012) emphasized the need for nitrate removal from RAS because chronic nitrate toxicity could damage growth rates, affected tissue structure and all body composition. As a result, additional processes were introduced such as denitrification reactors (Martins et al., 2009a, Martins et al., 2009b, Van Rijn et al., 2006) and sludge thickening technologies (Ebeling et al., 2006, Sharrer et al., 2009) to control nitrate and phosphate level in RAS.

The Integrated Multi-Trophic Aquaculture (IMTA) or zero waste aquaculture systems are the aquaculture with mixed trophic levels, both animal and plant. IMTA ensures that by-products (waste) from one aquatic species could be used as inputs (nutrients or food) for another species. One of the important concepts of IMTA is that aquatic animals and plants in the system must provide an advantage to the system and/or have substantial economic value such as; improve water quality, reduce feed costs and/or direct sale. In general, agriculturalists usually combine aquatic animals (e.g., fish, shrimp) with inorganic extractive (e.g., kelp) and organic extractive (e.g., bivalve) aquaculture to stimulate environment remediation (biomitigation), and stabilize the economics of the system (improved output, lower cost, product diversification and risk reduction) while maintaining social acceptability (better management practices). This, in turn, minimized the amount of wastes from this aquaculture system. For example; the cultivate the Tiger Catfish, *Pseudoplatystoma punctifer* with detritivorous and zooplanktivorous fishes (Gilles et al., 2014). Examples of IMTA as summarized in Table 2.6 illustrate that the implementation of this IMTA concept could improve growth/productivity of aquatic animal and enhance nitrogen nutrient removal.

Nitrogen compounds in the water from aquaculture activity, on the other hand, can be used as fertilizer for the growth of both microalgae and macroalgae. The implementation of microalgae and macroalgae (or aquatic plants) for the water treatment can greatly reduce the operating costs of water treatment. Microalgae, on the other hand, provide several benefits, not only on their high-value extracts, but also they serve as food for zooplankton and larval fish, etc. The macroalgae or aquatic plants can also pose some economical benefits as some of the species can be sold as decorating aquatic plants in aquariums. In addition, some macroalgae are rich in nutrients and can be used as an animal food supplement. Table 2.7 shows nitrogen treatment rate when using microalgae. Literature shows that microalgae can uptake nitrogen compounds at the rate of 22-330 mgNH_3-N/L/day, but the uptake rate of the macroalgae is unfortunately not reported.

Table 2.1 Accumulation behavior of lipid, protein and carbohydrate in *Chlorella* microalgae

References	Strain	Parameter												Biochemical composition (%)		
		Initial cell concentration		Medium	Air flow rate (mL/min)	Light intensity (Lux)	Photoperiod (L/D, h)	Temp. (°C)	pH	Cultivation time (Day)	Maximum cell concentration		μ (1/Day)			
		Cell/mL	g/L								Cell/mL	g/L		Total lipid	Protein	Carbohydrate
(Seyfabadi et al., 2011)	*Chlorella vulgaris*	$0.9x10^5$	n.r.	Zehnder	180	2,775	8:16	25±0.5	n.r.	10	$20x10^6$	n.r.	0.6±0.02	n.r.	33±2.0	n.r.
							12:12			9	$25x10^6$		0.69±0.03		34±2.5	
							16:8			8	$30x10^6$		0.81±0.04		36±2.2	
						4,625	8:16			10	$25x10^6$		0.7±0.04		35±2.3	
							12:12			8	$70x10^6$		0.83		38±3.5	
							16:8			8	$110x10^6$		1±0.02		43±3.0	
						7,400	8:16			7	$30x10^6$		0.8±0.04		40±3.5	
							12:12			7	$60x10^6$		1.02±0.03		42±4.5	
							16:8			7	$90x10^6$		1.13±0.04		46±3.7	
(Phukan et al., 2011)	*Chlorella sp.* MP-1	n.r.	n.r.	BG-11	n.r.	1,200	16:08	26 ± 2	n.r.	n.r.	n.r.	0.824	n.r.	28.82± 0.72	43.22 ± 0.33	9.46± 0.25
				Modified Chu 13								0.42		n.r.	n.r.	n.r.
				BBM								0.51				
				Basal								0.61				
(Yeh et al., 2010)	*Chlorella vulgaris* ESP-31	n.r.	0.015	Basal	300 rpm	5 (W/m2)	n.r.	25	6.2	3	n.r.	0.14	0.64±0.03	30-40	25-30	6-10
						9 (W/m2)						0.17	1.32±0.05			
						18 (W/m2)						0.17	1.61±0.08			
						30 (W/m2)						0.14	1.3±0.05			
						42 (W/m2)						0.14	1.1±0.07			
(Lv et al., 2010)	*Chlorella vulgaris*	n.r.	0.05	No-name	1,200	4,440	24:0	25	n.r.	1	n.r.	0.13	0.96	19	68	19
										2		0.3	0.90	15	45	44
										3		0.58	0.82	19	20	60
										4		0.7	0.66	21	11	67
(Widjaja et al., 2009)	*Chlorella vulgaris*	n.r.	n.r.	Modified Fitzgerald	6,000 (CO_2 0.03-3.33 %)	2,220		22	6.7-7.2	20	$13x10^6$	0.86	n.r.	29.53	n.r.	n.r.

n.r. = data not reported

Table 2.1 (cont.) Accumulation behavior of lipid, protein and carbohydrate in *Chlorella* microalgae

References	Strain	Parameter											Biochemical composition (%)		
		Initial cell concentration		Medium	Air flow rate (mL/min)	Light intensity (Lux)	Photoperiod (L/D, h)	Temp. (ºC)	pH	Cultivation time (Day)	Maximum cell concentration		μ (1/Day)			
		Cell/mL	g/L								Cell/mL	g/L		Total lipid	Protein	Carbohydrate
(Ramazanov & Ramazanov, 2006)	*Chlorella pyrenoidosa* 82T	$10x10^6$	n.r.	Minimal	5,000	66,600	12:12	28-30	n.r.	9	$1000x10^6$	n.r.	0.51	22.2 ± 1.3	50.2 ± 3.2	14.6
				Minimal without N						3			1.54	18.9 ± 2.1	n.r.	43.5
	Chlorella pyrenoidosa STL-PI			Minimal						9	$1200x10^6$		0.53	25.2 ± 1.2	62.5 ± 4.2	8.6 ± 2.1
				Minimal without N						3			1.60	38 ± 2.3	n.r.	24 ± 2.2
(Teoh et al., 2004)	*Chlorella* UMACC 237	n.r.	n.r.	BBM	n.r.	3,108	12:12	4	n.r.	n.r.	n.r.	n.r.	0.16	22	30	10
								6					0.19	15	78	10
								9					0.23	22	75	10
								14					0.24	13	35	10
								20					0.26	27	45	10
								30					0.19	11	29	8
(Belkoura et al., 1997)	*Chlorella* sorokiniana	n.r.	n.r.	n.r.	n.r.	10,360	n.r.	25	n.r.	n.r.	n.r.	n.r.	n.r.	13	28	20
						5,180		30						25	33	21
						10,360								18	37	31
						10,360		35						18	40	30
(Brown & Jeffrey, 1992)	*Chlorella prototthecoide* CS-41	n.r.	n.r.	MBL/NB_2	n.r.	5,550	n.r.	20	n.r.	n.r.	$2.38x10^6$	0.064	n.r.	12.8	25.6	10.8
	Chlordla sp. CS-247			F/2				20			$3.88x10^6$	0.031		14.75	15.3	13.55
	Chlordla sp. CS-195			F/2				27			$7.35x10^6$	0.031		17	19	5.94

n.r. = data not reported

Table 2.2 Accumulation behavior of lipid, protein and carbohydrate in other microalgae

Reference	Strain	Parameter										Biochemical composition (%)		
		Initial cell concentration		Medium	Air flow rate (mL/min)	Light intensity (Lux)	Temp. (°C)	pH	Cultivation time (Day)	Maximum cell concentration		μ (1/Day)			
		Cell/mL	g/L							Cell/mL	g/L		Total lipid	Protein	Carbohydrate
(Sayegh & Montagnes, 2011)	*Nannochloropsis* sp. (N)	n.r.	n.r.	F/2	n.r.	7,400	15	n.r.	n.r.	n.r.	n.r.	0.5	6.97	13.94	3.49
							20					1	24.92	20.76	14.53
							25					0.5	15.40	35.94	20.54
							30					0.8	40.35	20.17	30.26
	Isochrysis galbana (D)	n.r.	n.r.	F/2	n.r.	7,400	15	n.r.	n.r.	n.r.	n.r.	0.55	4.49	5.99	5.24
							20					0.61	10.01	14.02	16.02
							25					0.66	36.25	15.11	22.66
							30					0.72	36.90	6.15	24.60
(Mazzuca Sobczuk & Chisti, 2010)	*Choricystis minor*	n.r.	n.r.	BG-11	1,580 (CO_2 4.7 %V/V)	40,700	20	6	n.r.	n.r.	n.r.	n.r.	20.5 ± 0.8	n.r.	n.r.
							25						23.3 ± 2.3		
							30						21.1 ± 0.9		
(Yilmaz et al., 2010)	*Spilurina platensis* (Freshwater)	$7.6x10^6$ (filament/L)	n.r.	Schlosser	n.r.	n.r.	24.6-27.8	8.78-9.82	11	$10.4x10^6$ (filament/L)	n.r.	n.r.	7.44-9.38	49.97-57.47	24.95-27.41
(Gireesh et al., 2008)	*Chaetoceros calcitrans f. pumilus*	n.r.	n.r.	F/2	n.r.	37,000	20	n.r.	n.r.	$(1.8\text{-}5.9)x10^6$	n.r.	2.2-3.1	12-18	39-50	11-23
							25			$(2\text{-}6.3)x10^6$		1.7-4.5	12-24	25-59	13-24
							30			$(1.7\text{-}6.5)x10^6$		1.2-3.2	8-11	31-61	5-21
(De la Pena, 2007)	*Amphora* sp.	n.r.	n.r.	F/2MTM	n.r.	843	n.r.	n.r.	8	n.r.	n.r.	n.r.	81.5	600 pg/cell	58 pg/cell
(Takagi et al., 2006)	*Dunaliella tertiolecta*	n.r.	n.r.	Modified NORO	250 (CO_2 3 %)	11,100	30	8	10	n.r.	0.92 ± 0.02	n.r.	63.5±1.0	n.r.	n.r.
(Sánchez et al., 2000)	*Isochrysis galbana*	n.r.	n.r.	Ben-Amotz	500	14,800-16,000	15	8	5	n.r.	0.11	0.38	n.r.	37	n.r.
(Renaud et al., 1991)	*Nannochloropsis oculata* CS-179	n.r.	n.r.	Walne	n.r.	7,570	25±1	n.r.	n.r.	$7.09x10^6$	n.r.	n.r.	35.97	45.93	2.6
	Isochrysis sp. CS-177					10,116				$9.47x10^6$			38.63	36.77	10.57

n.r. = data not reported

Table 2.3 Advantages and disadvantages of biological nitrification systems

Nitrification system	Advantage	Disadvantage
Rotating Biological Contactor (RBC)	Short contact periods are required because of the large active surface. RBCs are capable of handling a wide range of flows. Sloughed biomass generally has good settling characteristics and can easily be separated from the waste stream. Operating costs are low because little skill is required in plant operation. Short retention time. Low power requirements. Elimination of the channeling to which conventional percolators are susceptible. Low sludge production and excellent process control.	Requirement for covering RBC units in northern climates to protect against freezing. Shaft bearings and mechanical drive units require frequent maintenance.
Trickling filters	Simple and reliable process that is suitable in areas where large tracts of land are not available for a Waste Stabilization Ponds (WSP) treatment system. Effective in treating high concentrations of organic material depending on the type of media used. Very efficient in removal of ammonia from wastewate. Appropriate for small- to medium-sized communities. Ability to handle and recover from shock loads. Relatively low power requirements. Level of skill and technical expertise needed to manage and operate the system is moderate. The cost to operate a trickling filter is very low.	Additional treatment may be needed for the effluent to meet strict discharge standards. Regular operators attention is needed. Relatively high incidence of clogging. Relatively low loadings required depending on the media. Limited flexibility and control in comparison with activated sludge processes. Potential for vector and odor problems.

Table 2.3 (cont.) Advantages and disadvantages of biological nitrification systems

Nitrification system	Advantage	Disadvantage
Submerged filter or fixed bed filter	Improve water quality as well as protecting pump. Optional filter media may be added for special purposes.	Much waste from filter may spill back into pond during cleaning. Solids collection and poor gas exchange in the submerged thin film filter
Fluidized bed reactor	Reduce the blockage of biological filter. Can treat wastewater from aquaculture which has high ammonia content. High surface area for bacterial growth.	Aeration system does not thoroughly.
Expandable media filters or Bead filter	Can improve wastewater which has amounts of sediment very well. Can separate the solids matter to prevent blockage effectively. Easy to expand or shrink.	High investment cost relative to the surface area of the filter. Wastewater does not treated thoroughly.
Airlift reactor	Good mixing and close contact between three phase, liquid, gas and solid Sufficiently high oxygen transfer from the gas to the liquid	
Immobilized in porous carriers	Can prevent microorganisms from being washed out and a high sludge age can be obtained. Short start-up time	High experience operate
Membrant reactor	High pollutant removal and low sludge production Membrane reactor lower than in a conventioned sludge process	Material was expansive Needed to regenerate membrane Higher maintemance

Table 2.4 Operating detail of various types of nitrification processes

References	Nitrification rate (gN/m^2d)	Volume (m^3)	Packing	Flow rate (L/min)	Retention time (min)	DO (mgO_2/L)	pH	Temperature (°C)	Salinity ($^0/_{00}$)	NH_4 - N ($mgNH_4$-N/L)
Tricking filter										
(Greiner & Timmons, 1998)	0.94 -3.92	0.06	commercial	b.	-	>5.0	6.7	26.4	-	-
(Arbiv & van Rijn, 1995)	0.149	2	-	250	0.02	5.8-7.2	7.0-7.9	22.5-27	-	2
(Knösche, 1994)	0.4	-	-	b.	-	-	7	25	-	5
(Kamstra et al., 1998)	0.24-0.55	-	-	b.	-	7.0-8.0	7	22-24	-	-
(Singh et al., 1999)		2	polyethylene	150	0.005	5.75-6.92		23.19	-	-
(Nijhof, 1995)	0.22(38.2%)	3	sieve screen	3458.33	$2.49x10^{-5}$	7.4-8.2	7.0-7.5	25	-	0.5-5
(Otte & Rosenthal, 1979)	0.75	1.06	plastic foil fiter	83.33	$2.5x10^{-5}$	6.0-7.5	8.2	20	8	15
(Rogers & Klemetson, 1985)	0.012	0.04	slag	0.16	0.342	5.0-6.0	7.1-8.5	25.5-30.0	20	9.3
Submerged filter										
(Menasveta et al., 2001)	0.068	6	plastic ball	b.	-	-	7.5	-	-	2
(Bower & Turner, 1983)	91%	3	limestone	b.	-	-	8.32	25.3	30	6.23
(Shanableh & Hijazi, 1998)	1.5	-	polypropylene	b.	-	5.0-7.0	-	-	-	8.0-9.0
(MacMillan et al., 1994)	0.083	2.3	polyester and charcoal	7	328.57	-	8.0-8.4	22	27-31	10
(Davis & Arnold, 1998)	0.59	0.72	polypropylene	280	25.714	10.2	-	-	-	-
(Nijhof & Bovendeur, 1990)	0.28	3.5	plastic	b.	-	-	-	24	17-34	-
(Tschui et al., 1994)	0.48	-	biocarbon	3.0-4.0	-	-	-	10	-	-
	1.43	-	polystyrene	6.0-7.0	$1.97x10^{-5}$	-	-	10	-	-
	1.67	-	plastic	>10	$1.47x10^{-5}$	-	-	10	-	-
(Tseng et al., 1998)	0.23	0.72	plastic	b.	20	3.6	7.48-7.96	32	33	3.64
(Yang et al., 1989)	0.69	-	crush oyster shell	81	-	-	8	30	34-36	-
(Wickins, 1985)	0.43	-	plastic	0.083	-	-	-	28	20-34	0.2
Airlift reactor										
(Van Benthum et al., 1998)	1.25	0.003	basalt	0.008	240	>3	7.5	30	-	5
(Sakairi *et al.*, 1996)	1.3	0.0157	polyethyleimine	b.		5	8.1-8.4	28	-	1.3
(Seo *et al.*, 2001)	2	2.5	-	250	2880	5.2	7.8-8.2	25	-	
	4.33	1.7	basalt	7-8	-	-	-	-	-	4.37

b = operated batch type

Table 2.4 (cont.) Operating detail of various types of nitrification processes

References	Nitrification rate (gN/m^2d)	Volume (m^3)	Packing	Flow rate (L/min)	Retention time (min)	DO (mgO_2/L)	pH	Temperature (°C)	Salinity ($^0/_{00}$)	NH_4 - N ($mgNH_4$-N/L)
Fluidized-bed filter										
(Aurelio & Lawson, 1996)	-	170	polyethylene	-	-	5.3	7.98	30.4	-	-
(Skjølstrup et al., 1998)	-	53	-	-	-	-	7	17.6	-	2.2
CSTR										3.3
(Kim et al., 2000)	0.82	-	Ba-algenated	-	18	7.5-7.9	-	25	-	20
Sequency batch reactor										
(Sliekers et al., 2002)	0.15	2	-	-	1440	-	7.8	30	-	14
Rotating Biofilter Contactor (RBC)										
(Rogers & Klemetson, 1985)	0.06	0.04		0.08		5.0-6.0	7.8	25-30	20	10
(Aurelio & Lawson, 1996)	-	5.12	polyethylene	-	-	5.3	-	7.98	30.4	-
Biodrum										
(Wortman & Wheaton, 1991)	-	0.009	-	-	-	5.8-7.1	8	25	-	7.95
(Rogers & Klemetson, 1985)	0.05	0.04	slag	0.08	-	5-6	7.8	25-30	20	10
Ponds										
(Gross *et al.*, 2000)	0.7	-	-	-	-	-	-	-	-	5.9
Immobilized in porous carrier										
(Kim *et al.*, 2000)	8.2	-	alginate	-	18	-	-	55	-	-
(Seo et al., 2001)	70	-	-	-	720	-	-	25	-	10
Membrane biofilm reactor										
(Huang et al., 2001)	-	-	-	-	300	-	6.8-7.2	-	-	-
(Delgado et al., 2002)	-	170	-	-	-	-	8.5	-	-	-

b = operated batch type

Table 2.5 Operating detail of various types of denitrification processes

References	Denitrification rate (mgNO3-N/L.min)	Volume (L)	Packing type	Flow rate (L/min)	Retention time (min)	DO (mgO_2/L)	pH	Temperature (°C)	Salinity ($^0/_{00}$)	NO_3-N ($mgNO_3$-N/L)
Fixed film column										
(Nalcaci et al., 2011)	0.0096	8.64	*G. verrucosa* and LMG	0.047	184.8	-	6.95-8.37	20	-	10
(Singhabhandu, 2001)	0.0124	-	Plastic bioballs	0.043	-	-	-	29±2	30	100
(Boley et al., 2000)	-	82.5	Polymer pellet	0.01	8,250	-	8	27-28	-	40
(Lee et al., 2000)	-	-	Glass bead	-	200	0.8-1.2	7-8	-	-	-
	0.02-0.025	60	Glass bead	-	-	< 1.5	-	-	-	80
(Balderston & Sieburth,	0.007	1.5	Limestone and Plastic	0.0075	200	< 1.2	-	20±1	18	100
Activated sludge tank										
(Otte & Rosenthal, 1979)	0.008	1060	-	2	530	6-7.5	7	22-26	8	1208
Fluidized bed column										
(van Rijn & Rivera, 1990)	0.2	131.5	Sand	5-40	12-13	< 0.2	7	27	-	50
Floating immoblilized				200						
(Sakairi et al., 1996)	1.44 per carrier	9	Cellulose carriers (3mm)	0.022	-	1	8	30	7.23	20
(Boley *et al.*, 2000)	0.02-2.77	82.5	Biodegradable polymer	0.003-0.01	8,250-27,500	-	6-8	20-25	-	5-40
Pond										
(Gross et al., 2000)	0.038 $g/m^2.d$	-	-	-	-	-	7-9	21-28	-	-

Table 2.6 Example of Integrated Multi-Trophic Aquaculture System (IMTA)

References	Cultured species	Culture densities	Volume	Water flow rate	Cultivation time	Aims	Productivity	N removal
(Samocha et al., 2015)	Shrimp (*Litopenaeus vannamei*); Seaweed (*Gracilaria tikvahiae*)	2.09 kg/m^3 2.96 kg/m^3	15 m^3 5 m^3	n.r.	7-18 days	G, N	11.75 g/m^2/d 98.6 gFW/m^2/d	72 g/m^2/d
(Waller et al., 2015)	Fish (*Dicentrarchus labrax L.*); Halophyte plant (*Tripolium pannonicum*) (*Plantago coronopus* L.) (*Salicornia dolichostachya*)	1.18 kg/m^3 0.17 kg/m^3	8 m^3 5.7 m^3	5.4 m^3/d	35 days	G, N	20 g/m^3/d 115.29 g/m^3/d	n.r. 0.16 g/m^2/d
(He et al., 2014)	Fish (*Sciaenops ocellatus*); Seaweed (*Gracilariopsis longissima*)	36 g/L 3;9 g/L	78 L 18;24.3 L	n.r.	30 days	G, N	0.026 g/L/d 0.164 g/L/d	n.r. 0.05mg/g/d
(Ben-Ari et al., 2014)	Fish (*Sparus aurata*); Seaweed (*Ulva lactuca*)	n.r. 1.33 kg/m^3	n.r. 12 m^3	0.64 m^3/h	26 days	N	n.r. 178 ± 71 g/m^2/d	n.r. 3.79 g/m^2/d
(Corey et al., 2014)	Fish (*Hippoglossus hippoglossus*): Seaweed (*Palmaria palmata*)	n.r. 2.95 kg/m^3	73.5 m^3 1.25 m^3	n.r.	1 year	G, N	n.r. 18.9 g/m^3/d	n.r. 0.28-0.56 gN/kgDW/d
(Robledo et al., 2014)	Shrimp (*Farfantepenaeus brasiliensis*); Seaweed (*Hydropuntia cornea*)	2.42 g/L 6 g/L	180 L 270 L	510 L/d	28 days	N	n.r. 0.03 g/L/d	n.r. 88.50%
(Abreu et al., 2011)	Fish; Seaweed (*Gracilaria vermiculophylla*)	n.r. 3.75 kg/m^3	n.r. 1.2 m^3	100 L/h 100 L/h	9 months	G,N	n.r. 0.7 ± 0.05 kg/m^2/month	n.r. 40.54 ± 2.02 g/m^2/month

n.r. = data not reported

Aim: G = Growth/production; N=Nitrogen compound removal; W= Water saving

Table 2.7 Nitrogen removal rate by microalgae

References	Strain	Type of reactor	Parameter								Removal rate (mg/L.day)		
			Initial cell. (g/L)	Medium	Air flow rate (mL/min)	Light intensity (Lux)	Temp. (ºC)	pH	Duration (Day)	Max. cell. (g/L)	µ (1/Day)	NH_3	NO_2	NO_3
(Hai et al., 2011)	*Chlorella* sp. USTB-01	Fermentor-helical photobioreactor (30 L)	0.4	Modified Fitzgerald	16,667	n.r.	30±1	6.5	3	4.9	0.69	330	n.r.	n.r.
(Yun et al., 1997)	*Chlorella vulgaris*	Photobioreactor (Bottle 0.2 L)	0.04	N_8	400	8140	27	Not control	4.9	1.72 & 1.49	0.68	22.08	n.r.	n.r.
(Yoshihara *et al.*, 1996)	NOA-113	Long tubular photobioreactor (4 L)	0.4 (g AFDW/L)	Modified F/2	150	(38 $W/m^{2)}$	25	6	6	2.4 (g AFDW/L)	0.12	n.r.	10	n.r.

n.r. = data not reported

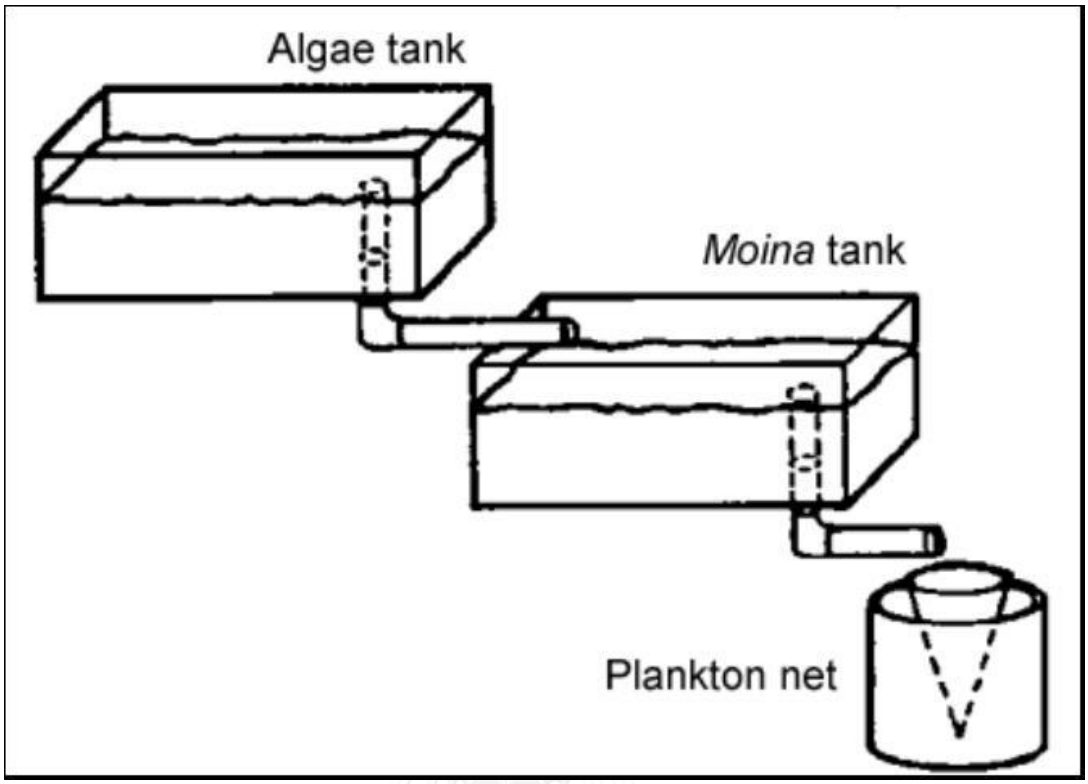

Figure 2.1 Tank arrangement for the separate culture of *Moina* and its food (Rottmann et al., 2004)

CHAPTER III
EXPERIMENTAL

This work was designed to give engineering detail for the design of a recirculating aquaculture system where wastes from one part are being consumed as feed for other organisms in the whole concocted ecosystem (Integrated Multi-Trophic Recirculating Aquaculture System, IMRAS) as shown in Figure 3.1. However, there are complexities in the relationships between the various components in the ecosystem which needs to be identified and optimized. Therefore the experiments were intentionally separated into 2 main parts. Part I (Algal nutrition adjustment) involves an in-depth study of the effect of several environmental parameters on the accumulation of nutritional components in a phytoplankton (*Chlorella* sp.). Part II (Outdoor cultivation) describes an outdoor growth of *Chlorella* sp. to be used as a feed for zooplankton (*Moina macrocopa.*) and multi-trophic recirculating aquaculture system, the investigation of the closed loop outdoor fish cultivation system, where *Chlorella* sp. and aquatic plants tanks takes part as a nitrogen conversion unit. Each part will be delineated as follows.

3.1 Effects of environmental conditions on nutritional productivity of *Chlorella* sp. (Chapter IV)

This experiment studied the various parameters, including temperature, light intensity, and aeration rate, which affected the growth and accumulation of valuable nutrition of microalga *Chlorella* sp. The experiment was conducted using 2 L photobioreactor (bubble column photobioreactor) placed in a controlled chamber where temperature and light intensity can be controlled at a certain level. The control chamber used in this experiment was made from clear acrylic to allow the visual observation of the ongoing phenomena. The control chamber includes twelve compact fluorescence light bulbs (20 Watts) as a light source and a temperature control elements. Compact fluorescence light bulbs will be mounted with acrylic sheet on the channel to be able to move in - out from the reactor, (to increase - decrease light

intensity, respectively). The temperature was controlled using an evaporative cooling system and heater system. In the evaporative cooling system, the water was vaporized to remove heat and lower the air temperature. The heater system is a simple heating bar to heat the air to a required temperature. Light intensity and temperature were controlled in the range from 10-30 kLux (± 0.1 kLux) and 30-40ºC (± 0.5ºC). A calibrated rotameter was used to control the volume of gas volumetric flow rate supplied to the system through a porous gas sparger at the base of photobioreactors in the range from 0.5-1.5 cm/s (± 0.05 cm/s). Details of how the experiment was conducted (Design of Experiments, DOE) are provided in Figure 3.2. The experiment was started by varying light intensity, and the best result was employed for the following experiments. The culture medium was the modified M4N (detail in Table 3.1) (Kumoro & Susanto, 2013, Sung et al., 1999). Samples were collected daily in order to analyze the growth. After cell harvest (6 days), microalgal biomass was collected in order to analyze for the accumulation of nutritional components including total lipid, protein, carbohydrate, and lutein. All experiments in this study were set as 6 sets to maintain statistical reliability at high level and to obtain a proper amount of dry weight for biochemical composition analysis. The experimental methods were discussed in the next step which can be summarized as follows.

3.1.1 Effect of temperature on growth and nutritional quality of the alga

1. Inoculate *Chlorella* sp. in 2 L glass bottles until the cell concentration of approximately 70-100 x 10^6 cell/mL
2. Sterilize all photobioreactors with steam (Autoclave) at 121ºC for 15 minutes
3. Add fresh water into all photobioreactors, culture medium together with the pure culture (from Step 1) and adjust the total volume to 2 L. Initial cell concentration are controlled at 1x10^6 cell/mL
4. Control air temperature constant at 30 ºC with a temperature controller
5. Adjust light intensity to approximately 10,000 Lux by moving light bulb on acrylic sheet into or out from the surface of the photobioreactor

6. Supply sterilized compressed air (treat with the air filter size 0.2 μm) through a porous gas sparger and control aeration rate at 1 cm/s (3.4 vvm)
7. Take samples once a day (add fresh water into the photobioreactor by the water level was beginning before taking samples) and count for the cell density using haemacytometer (mentioned in Section 3.4)
8. Harvest cells from all photobioreactors when the culture reaches its stationary phase
9. Isolate cells from water by centrifuge at a speed of 4,000 rpm for 15 minutes
10. Wash the cells with fresh water a few times before isolating the cells from the washed water by centrifuge again at a speed of 4,000 rpm for 15 minutes
11. Freeze and dry the algal biomass for a period of 2-3 days, powder cells derived from this step is dry cells
12. Analyze for moisture, ash, protein, fat and carbohydrates in the dry cells
13. Repeat the experiment from Steps 1 to 12, but change the temperature in Step 4 to 35, and 40 º C, respectively

3.1.2 Effect of light intensity on growth and nutritional quality of the alga

1. Repeat Experiments in Section 3.1.1 with the optimal temperature
2. Change the light intensity from 10,000 to 15,000 to 20,000 and 30,000 Lux

3.1.3 Effect of aeration rate on growth and nutritional quality of the alga

1. Repeat Experiments in Section 3.1.1 with the optimal temperature and light intensity
2. Change the aeration rate from 1.7 to 3.4 and 5.1 vvm (equivalent to superficial velocity of 0.5, 1 and 1.5 cm/s, respectively)

3.2 Design of combined closed loop aquaculture system (Chapter V)

Experiment in this section was a design study of the integrated multi-trophic recirculating aquaculture system. A prototype system used in this experiment was the cultivation system of fish, phytoplankton, zooplankton, and aquatic plants in a continuous circulating mode. Fish used in the experiment were *Oreochromis niloticus* (Nile tilapia) which was representative of farm fish culture for food. Phytoplankton (microalgae) and zooplankton used in the experiment were *Chlorella* sp. and *Moina macrocopa*, respectively. *Egeria densa*, *Ceratophyllum demersum*, *Hygrophila difformis*, *Vallisneria spiralis* and *Vallisneria Americana* were used as aquatic plants in this experiment. Order of flow began from the fish aquarium to phytoplankton or microalgae tank, zooplankton tank, and aquatic plants tank (see Figure 3.3 for flowchart). The use of microalgae and the aquatic plants was to remove nitrogen compounds, e.g. NH_3, NO_2^-, and NO_3^-, generated as a waste from fish and zooplankton. This worked together with the nitrification and denitrification bacteria naturally resided in the system. The treated water in the final water plants tank was returned back to the fish aquarium. Experimental procedure follows.

3.2.1 System setup

In this work, a duplicate cultivation of sex-reversed male Nile tilapias (*Oreochromis niloticus*) was carried out in the control and treatment systems. In the control cultivation, fish were cultured in the oval shape opaque fiber (diameter 0.8 m, depth 0.4 m, working volume of 200 L) where the water was not treated (representing typical cultivation practice). On the other hand, the treatment system consisted of a

series of tanks connected together as shown in Figure 3.3. This system, called Integrated Multi-trophic Recirculating Aquaculture System (IMRAS), included fish tank (Section 3.2.2), phytoplankton tank (Section 3.2.3), zooplankton tank (Section 3.2.4) and aquatic plants tank (Section 3.2.5). The water in the aquatic plants tank was pumped to the fish tank using a submersible pump. An overflow conduit was installed from the fish tank to the phytoplankton tank and the aquatic plants tank. A valve was provided to allow a partial overflow of the water from the fish tank to the phytoplankton tank. This valve remained open until the phytoplankton tank was filled up where the valve was shut and the tank was then operated in a batch mode for the cultivation of *Chlorella* sp. as described in Section 3.2.3. Once the stationary growth phase was reached, the phytoplankton culture was transferred to the zooplankton tank as a feed for *Moina macrocopa* and this is described in Section 3.2.4, and the overflow valve was turned on again. A part of the water from the fish tank continuously overflowed to the aquatic plants tank before being pumped back to the fish tank to finish the cycle. The water pumping rate was set at 700 mL/min which is equivalent to a recirculation with a hydraulic retention time of one day.

During the experiment, the growth rates of Nile tilapia, *Chlorella* sp., *Moina macrocopa*, and aquatic plants, along with the water quality such as concentration of ammonia, nitrite, nitrate, phosphate, alkalinity, temperature and dissolved oxygen (DO) were measured following the standard methods for water and wastewater analysis (APHA, 2005).

3.2.2 Fish tank (Nile tilapia)

The fish tank was made from fiber glass with the working volume of 1,000 L (dimension: length 1.7 m, width 1 m, depth 0.6 m). An air compressor (LP100, Resun) was used to provide the dissolved oxygen at the level greater than 5 mg/L and also to promote liquid circulation. This level of dissolved oxygen was reported to be enough for growth of this fish (Riche & Garling, 2003).

The experiment started with three different fish stockings, i.e. 20, 25 and 50 fish/m^3, with the initial average weight of 2 g/fish. Feeding was provided twice a day

(morning and evening) each at 5% of the total fish weight. The feed composition was 28% crude protein, (nitrogen and phosphorus content were 5.24 and 1.14% of dry weight matter). This amount of protein was recommended as suitable for Nile tilapia by Ribeiro *et al.* (2006). Note that during the experiment, the weight of all fish was recorded at every 28 days. The experiment was carried out for 112 days before harvest. At the end of the experiment, the weight gain (g), daily weight gain (g/d), feed conversion ratio (FCR) and survival rate (%) are calculated as describe in Section 3.3. In addition, Nile tilapia sample was analyzed for its moisture content, dry weight matter and chemical compositions in order to calculate nitrogen and phosphorus mass balances.

3.2.3 Phytoplankton tank (*Chlorella* sp.)

Chlorella sp. was cultivated with the M4N medium (Lee *et al.*, 2011, Sung *et al.*, 1998) without KNO_3 and K_2HPO_4 as N and P sources were obtained from the fish excrete. The culture tank was made from transparent glass with a working volume of 100 L (length 1.5 m, width 0.28 m, depth 0.24 m). The initial biomass density was 0.01 g/L (10^6 cell/mL) with a continuous aeration rate of 0.1 vvm (10 L/min). Sample was collected once a day to measure dry weight. *Chlorella* sp. was harvested as it entered the stationary growth phase (generally after 4 days of cultivation) and was used as a feed for *Moina macrocopa*.

3.2.4 Zooplankton tank (*Moina macrocopa*)

The zooplankton tank was made from a fiber glass with a working volume of 100 L (diameter 0.8 m, depth 0.2 m). *Chlorella* sp. as harvested from Section 3.2.3 was used in the cultivation of *Moina macrocopa* with the initial concentration of 0.1 g/L. An aeration rate of 0.01 vvm (1 L/min) was supplied at the center of the tank in order to increase the level of dissolved oxygen in water and also to prevent cell precipitation. *Moina macrocopa* generally spent 4 days to reach its stationary phase in which it was harvested with 150 μm plankton net. The culture water after cell removal was sent to treat in the aquatic plants tank (Section 3.2.5).

3.2.5 Aquatic plants tank

The aquatic plants tank was the major component of the treatment because most nitrogen and phosphorus compounds were removed here and used for the growth of the aquatic plants. Moreover, aquatic plants could capture the suspending sediment from the fish tank, which helped maintain not only the level of nitrogen and phosphorus, but also the clarity of the water in the system. The aquatic plants tank was made from fiber glass with total working volume 800 L (length 1.4 m; width 1.2 m; depth 0.53 m). The tank was operated under outdoor condition to utilize sunlight as an energy source for the growth of the aquatic plants.

In this tank, soil (120 kg) was filled at the bottom at the height of 5 cm, where the water depth was 48 cm. Several aquatic plants, i.e. *Hygrophila difformis, Vallisneria spiralis, and Vallisneria Americana*, each with the initial fresh weight of 100 g were planted in the soil, whereas *Egeria densa* and *Ceratophyllum demersum* (100 g each) were floated on the water surface. Aquatic plants were harvested every 14 days such that the remaining weight of each aquatic plant was equal to the initial fresh weight (100 g each). The harvested aquatic plants were analyzed for their dry weights, moisture contents, nitrogen and phosphorus balances.

The experimental methods can be summarized as follows:

Preparation of apparatus

1. Fill water into the fish tank to a volume of 1 m^3, and supply air (0.05 vvm) through a porous sparger
2. Fill water into *Chlorella* sp. and *Moina macrocopa* tanks to a volume of 0.1 m^3, and supply air (0.1vvm) through a porous sparger
3. Add soil in the aquatic plants tank and supply water to a volume of 0.8 m^3, leave the water in the tank for two days for small particles sedimentation

5. Grow the aquatic plants, e.g. *Hygrophila difformis*, *Ceratophyllum demersum*, *Vallisneria spiralis*, and *Egeria densa* in the aquatic plants tank

Recirculation aquaculture system

1. Add 20 Nile tilapias into the fish tank and record the weight of all Nile tilapias
2. Feed twice a day to fish tank using the feeding ratio of 5% of the weight of fish
3. Pump continuously of water (700 L/min) from aquatic plants tank to the fish tank. Water level in the fish tank was controlled by the overflow in the tank. Water was overflowed from fish tank to phytoplankton tank as batch flow (close/open valve) and to aquatic plants tank as continuous flow through 20 mm diameter of pvc pipes
4. Cultivate *Chlorella* sp. in the phytoplankton tank (initial cell concentration = $5x10^6$ cell/mL)
5. After culture of *Chlorella* sp. for 4-5 days, phytoplankton was overflowed to zooplankton tank
6. Add 10 g of *Moina macrocopa* in zooplankton tank
7. After culture of *Moina macrocopa* for 4 days, *Moina macrocopa* was harvested with 150 µm plankton net, which this filtrate flowed to aquatic plants tank again
8. Take samples every day for cell counting and analysis of the amount of NH_3, NO_2^-, and NO_3^-
9. Repeat the experiment from Steps 1 to 8, but change the amount of Nile tilapia in Step 1 to 25 and 50, respectively

3.3 Analysis

3.3.1 Determination of microalga concentration

The microalga concentration can be determined using a normal blood cell counting slide, Haemacytometer. The depth of the counting grid and the area are 0.1 mm and 0.04 mm^2 respectively. The cell concentration can be determined as follows:

1. Clean the counting slide and cover glass
2. Fill the slide with sample
3. Cover the slide with cover glass, avoid the presence of bubbles.
4. Count the cell in 25 small squares on the grid (25 small squares per 1 large square)
5. Repeat Steps 1–4 two times
6. Calculate the cells number, using Equation 3.1:

$$N = \frac{1}{25} \times \frac{1}{4} \times n \times 10^6 \tag{3.1}$$

where

N = cells concentration (cells/mL)

n = number of cells on 25 small squares (cells)

3.3.2 Determination of specific growth rate

The specific growth rate can be calculated from Equation 3.2 as follows:

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \tag{3.2}$$

where

μ = specific growth rate (1/h)

N_1 = cells concentration at t_1 (cell/mL)

N_2 = cells concentration at t_2 (cell/mL)

t_1 = first sampling time (h)

t_2 = second sampling time (h)

3.3.3 Determination of productivity

The productivity of the phytoplankton and zooplankton were calculated from Equation 3.3 as follows:

For batch and semi-continuous cultivation:

$$P = \frac{N_2 - N_1}{t_2 - t_1} \times V \times 1000 \quad (3.3)$$

where

P = productivity (cell/h)

N_1 = cells concentration at t_1 (cell/mL)

N_2 = cells concentration at t_2 (cell/mL)

t_1 = first sampling time (h)

t_2 = second sampling time (h)

V = harvest volume (L)

For continuous cultivation:

$$P = N \times F \quad (3.4)$$

where

P = productivity (cell/h)

N = effluent cell concentration (cell/mL)

F = volumetric flow rate of fresh medium (mL/h)

3.3.4 Determination of specific productivity

The specific productivity for the cultivation of the phytoplankton and zooplankton can be calculated from Equation 3.5:

$$SP = \frac{P}{V} \quad (3.5)$$

where

SP = specific productivity (cell/L.h)

P = productivity (cell/h)

V = harvest volume (L)

3.3.5 Determination of total lipid

Biomass of microalgae in photobioreactor was harvested by centrifugation at 4,000 rpm, 10°C for 15 min (Kubota 7820). The cells were washed twice with deionized water. After drying the samples using freeze drier, the samples were pulverized in a mortar and extracted using mixture 2:1 (v/v) of chloroform:methanol (Bligh & Dyer, 1959) by soxhlet method. About 180 mL of solvents were used for every gram of dried sample in extraction step. The weight of total lipids was measured after removing solvent by rotary evaporator (R-215, Buchi, Switzerland). The residue in evaporation flask from the evaporation step was weighed as total lipids.

3.3.6 Determination of protein

Total nitrogen content of microalgal biomass was detected by an elemental analyzer (Perkin Elmer PE2400 Series II). The protein concentration of microalgae was estimated from the obtained nitrogen content according to the correlation reported in the literature, i.e. protein concentration = nitrogen content x 4.44) (López *et al.*, 2010).

3.3.7 Determination of carbohydrate

Total carbohydrate concentration of microalgal biomass was determined by the anthrone-sulfuric acid method and compared with D+ glucose standard (Mirón *et al.*, 2002).

3.3.8 Determination of moisture

The moisture content of microalgal biomass was determined from the masses of microalgae before and after water evaporation. This method follows the procedure set out in the ASTM D 1762-84 (ASTM, 2013)

3.3.9 Determination of ash

Ash determination procedure according to Sluiter *et al.* (2008) which is substantially similar to ASTM Standard Method Number E1755-01 (Standard Method for the Determination of Ash in Biomass) was used to determine ash content in microalgae biomass.

3.3.10 Determination of lutein

Biomass was mixed with KOH and ethyl alcohol at the ratio of 1:0.6:10 (w/w/v). The mixture was shaken for 4 h after which ethyl alcohol (50 mL) was added. Lutein was extracted by solvent extraction with separatory funnel under dark place. Diethyl ether (80mL) and Na_2SO_4 (100 mL) were added. Low density lutein liquid was evaporated. Lutein was dissolved in ethyl alcohol where its quantity was analyzed by UV-VIS spectrophotometer (Agilant carry 60) at the wavelength of 478 nm.

3.3.11 Determination of weight gain, daily weight gain, feed conversion ratio (FCR) and survival rate of Nile tilapia

$$\text{Weight gain (g)} = \text{Final wet weight (g)} - \text{Initial wet weight (g)} \quad (3.6)$$

$$\text{Daily weight gain (g/d)} = \frac{\text{Weight gain (g)}}{\text{Cultivation time (d)}} \quad (3.7)$$

$$\text{FCR} = \frac{\text{Total amount of fish feed fed (g)}}{\text{Total wet weight gain (g)}} \quad (3.8)$$

$$\text{Survival rate (\%)} = \frac{\text{Total number of fish at final (fish)}}{\text{Total number of fish at initial (fish)}} \times 100 \quad (3.9)$$

Table 3.1 Modified M4N medium (Kumoro & Susanto, 2013, Sung et al., 1999)

Composition	Concentration (mg/L)
KNO_3	5,000
$MgSO_4.7H_2O$	2,500
KH_2PO_4	1,250
NaFeEDTA	14
H_3BO_3	2.86
$MnSO_4.7H_2O$	2.50
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
Na_2MoO_4	0.02

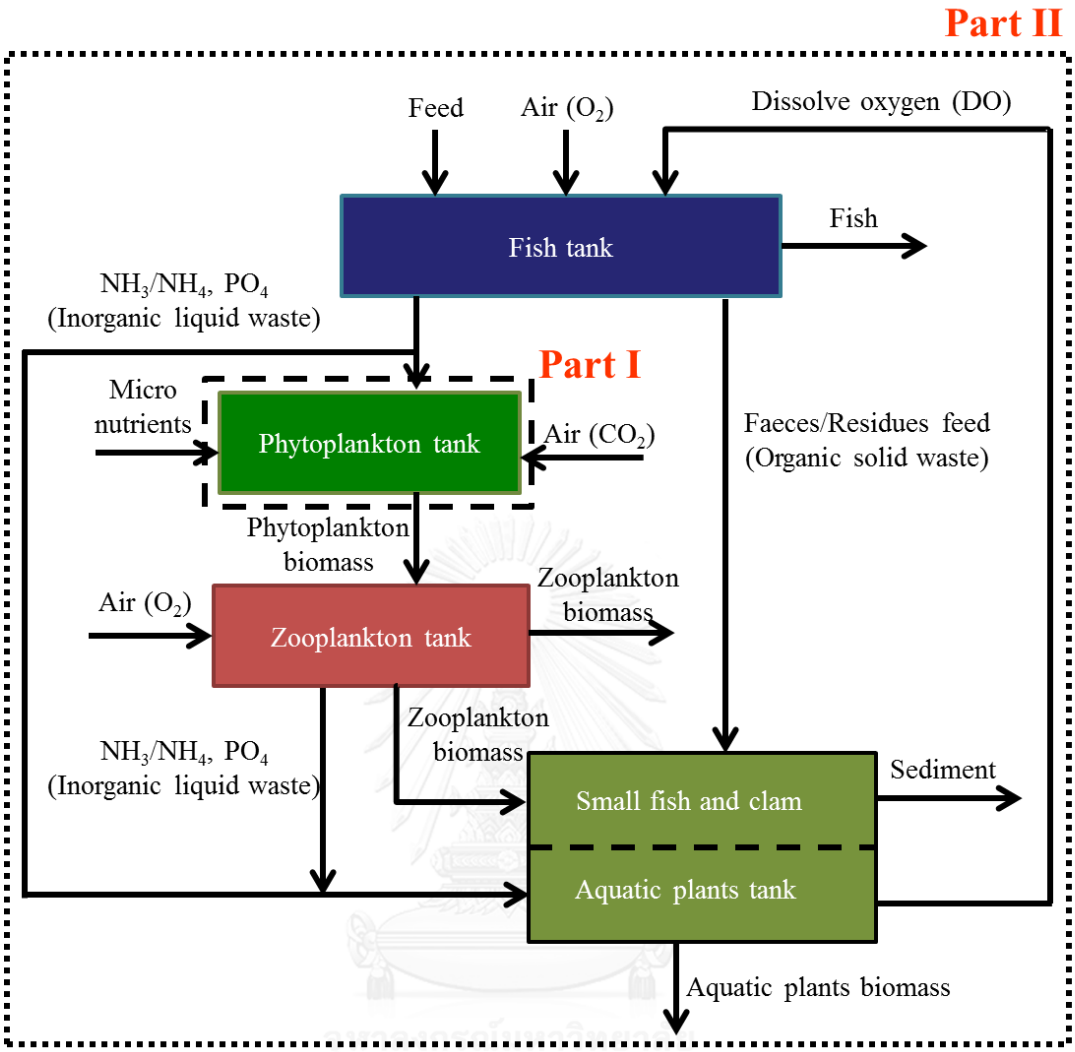

Figure 3.1 Diagram of Integrated Multi-Trophic Recirculating Aquaculture System (IMRAS) in this works.

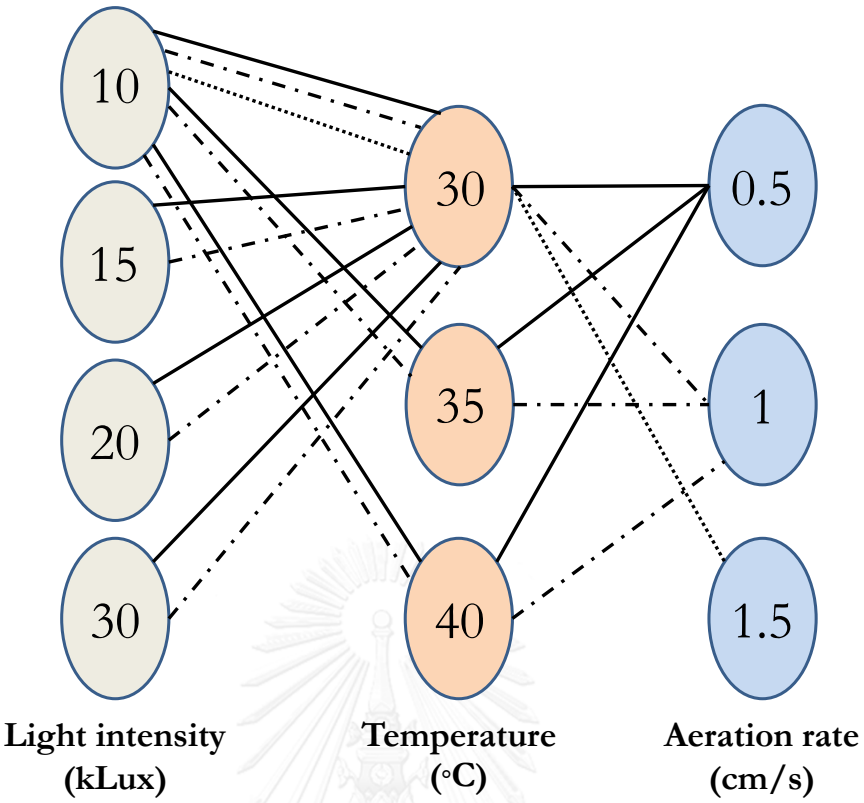

Figure 3.2 Details of how this experiment was conducted: The different line type indicates how the matching between the various parameters was investigated.

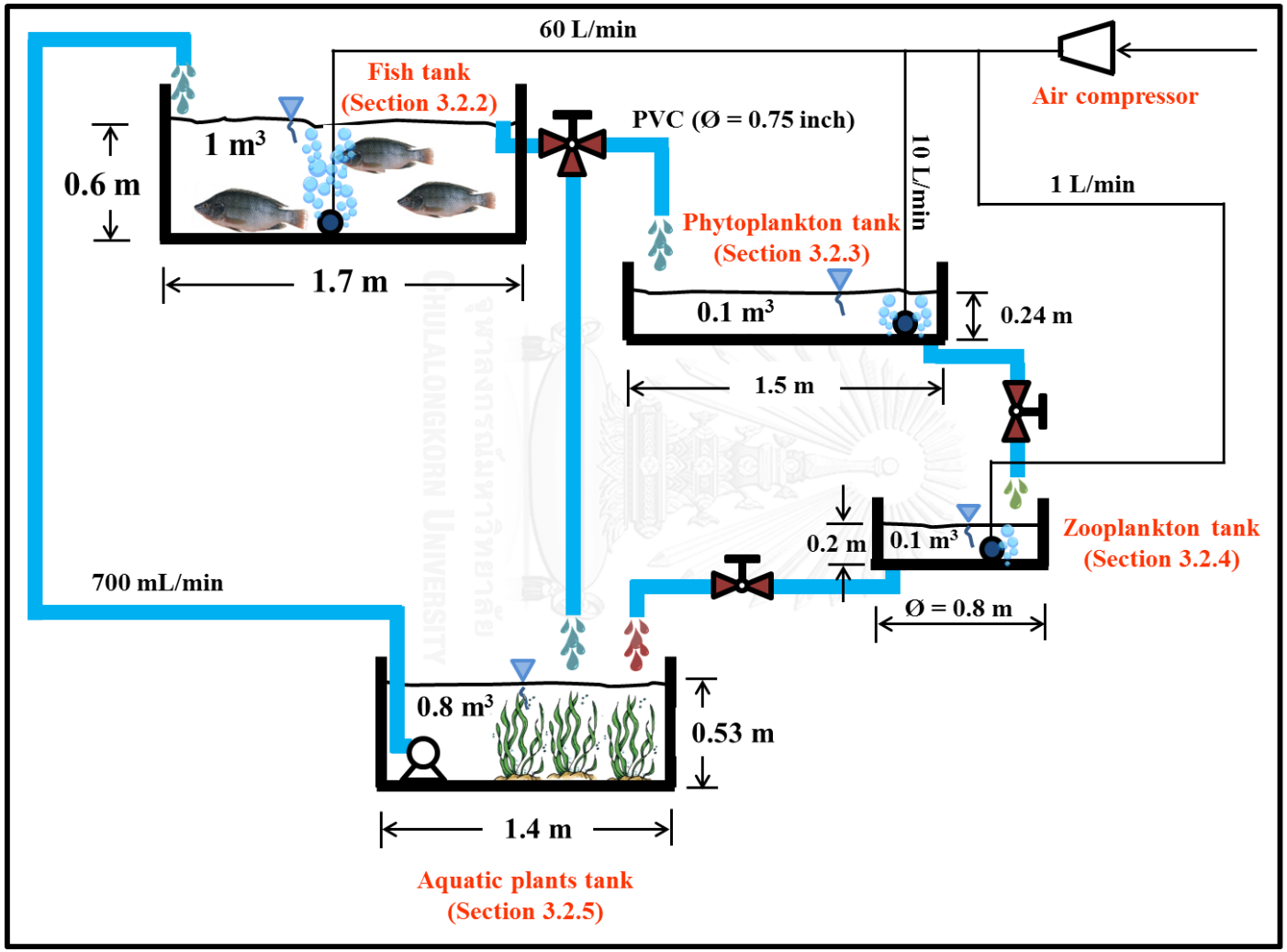

Figure 3.3 Integrated multi-trophic recirculating aquaculture system setup

Chapter IV

Manipulation of biochemical compositions of *Chlorella* sp.

4.1 Introduction

Thailand also has its climate condition which is considered most suitable for the cultivation of several algal cultures, especially *Chlorella*. Due to the variety of applications, *Chlorella* can also be grown under different growth conditions to enable the accumulation of various specific components as describe in Chapter I. Nevertheless, most previous studies had focused only on effects of various parameters on the growth of the alga while very few researches have worked on the induction effects of various growth parameters on the quality of the biomass. This work, hence, aimed to study the effect of several environmental parameters (light intensity, temperature, and aeration rate) on the accumulation of nutritional components and lutein production in a green microalgae *Chlorella* sp. The results of this work are described below:

4.2 Lipid productivity

The cultivation of microalgae *Chlorella* sp. was conducted under several conditions. The alga seemed to grow best at the light intensity of approximately 10 kLux, temperature of 35°C and aeration at superficial gas velocity of 1 cm/s which yielded the highest lipid productivity of 37 mg/L/d. Moving away from this optimal condition caused a decrease in lipid productivity, especially at high temperature, e.g. 38-40°C as shown in Figure. 4.1 where *Chlorella* grew considerably slowly (the raw data of this experiment are shown in Appendix). This corresponded well with the findings in literature which indicated that temperature higher than 37°C was not suitable for the growth of this algal species (Li et al., 2013), and the suitable temperature range should be around 25-30°C (Buchert et al., 2012, Lu et al., 2013, Oh et al., 2010, Sydney et al., 2010, Xie et al., 2012). Manipulating light intensity could facilitate the accumulation of lipid quite effectively. For instance, controlling the light intensity at 15 kLux could help enhance the lipid productivity to 36 mg/L/d when

Chlorella sp. was cultivated at 30°C and aeration rate about 1 cm/s compared to 30 and 26 mg/L/d at light intensity of 20 and 10 kLux, respectively. In some cases, providing high light intensity might help the alga to better utilize light as a compensation for the poor circulation. This was observed when the aeration was poorly fixed at 0.5 cm/s (instead of 1 cm/s) and the lipid productivity could still be maintained at as high as 35 mg/L/d by increasing light intensity from 15 to 25 kLux (see Figure 4.2).

4.3 Protein productivity

Figure 4.3 illustrates that the highest protein productivity (89 mg/L/d) was achieved at 10 kLux, 35°C, and 1 cm/s (aeration). Protein productivity decreased significantly to 53 and 29 mg/L/d if temperature changed to 30 and 40°C, respectively. This was due to two major reasons. First, biomass productivity was the highest at 35°C at 195 mg/L/d. This dropped slightly to 133 mg/L/d at 30°C, but significantly dropped to 57 mg/L/d at 40°C. Secondly, the protein content at 35°C was relatively high at 45.6%wt when compared to 39.8% at 30°C. Although the maximum protein accumulation of 50.8% occurred at 40°C, the productivity was extremely low due to the poor growth rate. Figure 4.4 demonstrates further that inducing protein accumulation might be achieved by providing high light intensity at high circulation rate. For instance, increasing light intensity and aeration velocity from 15 to 30 kLux and 1 to 1.5 cm/s could notably enhance the protein productivity from 53 to 74 mg/L/d.

4.4 Carbohydrate productivity

Figure 4.5 illustrates that the highest carbohydrate productivity of 60 mg/L/d could occur at two ranges of light intensity, i.e. at between 16 and 22 kLux, and at 30 kLux, both at 30°C and aeration velocity of 0.5 cm/s. When temperature and aeration were fixed at 30°C and 0.5 cm/s and the light intensity was reduced to 10 kLux, carbohydrate productivity decreased to 31 mg/L/d. At this low light intensity, there seemed to be an optimal range of temperature that could provide high carbohydrate

content, i.e. between 33 to 36°C. Above and below this temperature range, carbohydrate productivity declined. At light intensity greater than 13 kLux, carbohydrate accumulated most at low temperature, and in this experiment, this was found at 30°C. Temperature greater than 33 or 34°C led to a decrease in carbohydrate productivity.

4.5 Lutein productivity

Lutein was among one of the most interesting biocomponents from *Chlorella* sp. considering its high price and the financial return rate. Lutein accumulation was found to change with culture condition in a similar fashion to other nutritional compounds where the highest lutein productivity was 0.9 mg/L/d at 10 kLux, 35°C and aeration velocity of 1 cm/s. Lutein productivity seemed to decrease with increasing light intensity to greater than 10 kLux (Figure 4.6). It was observed clearly that a decrease in temperature to 30°C lowered lutein productivity down to 0.3 mg/L/d. In fact, lutein was most accumulated when cells were exposed to a relatively high light intensity. Surprisingly, this same condition also resulted in the highest cell productivity indicating that cells were not in a stress condition. Sánchez et al. (2008b) supported this finding and suggested that lutein was a primary metabolite of growth so the optimal condition for biomass productivity was the same with the optimal condition for lutein productivity.

Adjusting aeration could be a crucial factor for lutein accumulation (Figure 4.7) whereas lutein decreased with aeration above or below 1 cm/s. To maintain high lutein productivity, temperature had to be controlled within the range of 33-37°C, aeration 0.8-1.2 cm/s, and with light intensity of 10 kLux. At these conditions, lutein productivity was in the range of 0.8-0.9 mg/L/d (270 mg/m^2/d) which was about 16 times higher than that from marigold (Palumpitag et al., 2011). Table 4.1 illustrates the comparison of lutein yields from the various types of cultures which indicates that lutein from *Chlorella* was still not so high when compared with other algal species like *Chlorococcum citriforme*. However, *Chlorella* sp. is among the most common algal species in tropical area and the cultivation of such culture could be economically carried out which renders the production of lutein from such species more attractive.

4.6 Conclusions

This work demonstrated that nutritional composition accumulated during the cultivation of *Chlorella* sp. could be adjusted, although slightly in some cases, by manipulating typical culture conditions at its most appropriate level, such as temperature, light intensity and aeration velocity. The selection of operating conditions therefore needs to be carefully considered to ensure that the final quality of the product could be achieved. For this work, the various cultivating conditions for the different purposes were examined and the summary of such conditions along with the comparison between the reported biochemical compositions from *Chlorella* spp. and those obtained from this work are provided in Tables 4.2 and 4.3, respectively.

Table 4.1 The comparison of lutein yields from the various types of cultures

Biomass	Lutein content (mg/g dry biomass)	Lutein productivity (g/rai/day)	Lutein productivity (g/m^2/day)	Lutein productivity mg/L/d	References
Marigold	14.0	27	0.017	-	(Palumpitag *et al.*, 2011)
Muriellopsis sp.	4-6	160	0.10	-	(Blanco *et al.*, 2007)
	4.3	288	0.18	7.2	(Del Campo *et al.*, 2001)
	5.5	336	0.21	0.8-1.4	(Del Campo *et al.*, 2000)
Scenedesmus almeriensis	5.5	1,176	0.74	4.9	(Sánchez *et al.*, 2008b)
	4.5	464	0.29	-	(Sánchez *et al.*, 2008a)
Chlorella protothecoides	4.6	2,400	1.50	10.0	(Wei *et al.*, 2008)
Chlorella zofingiensis	3.4	816	0.51	3.4	(Del Campo et al., 2000)
Chlorococcum citriforme	7.2	6,048	3.78	25.2	(Del Campo et al., 2000)
Neospongiococcus gelatinosum	7.6	4,032	2.52	16.8	(Del Campo et al., 2000)
Chlorella sp.	4.9	432	0.3	0.9	This work

Table 4.2 The summary of the conditions for each nutritional component of *Chlorella* sp.

Major Component	Maximal productivity (mg/L/d)	Optimal condition			Algal biomass productivity (mg/L/d)	Minor biocomponent productivity (mg/L/d)			
		Temperature (°C)	Light intensity (kLux)	Aeration velocity (cm/s)		Lipid	Protein	Carbohydrate	Lutein
Lipid	37	35	10	1	196	-	89	45	0.9
Protein	89	35	10	1	196	37	-	45	0.9
Carbohydrate	61	30	30	0.5	185	33	68	-	0.4
Lutein	0.9	35	10	1	196	37	89	45	-

Table 4.3 The comparison between the reported biochemical compositions from *Chlorella* spp. and those obtained from this work

Strain	Operating parameter			Maximum biomass concentration (g/L)	Productivity (mg/L/d)			Reference
	Areation rate	Light intensity (Lux)	Temperature (ºC)		Lipid	Protein	Carbohydrate	
Chlorella vulgaris ESP-31	300 rpm	3,057	25	0.17	23	17	6	(Yeh et al., 2010)
Chlorella vulgaris	1200 (mL/min)	4,440	25	0.7	37	19	117	(Lv et al., 2010)
Chlorella vulgaris	6000 (mL/min)	2,220	22	0.86	13	-	-	(Widjaja et al., 2009)
Chlorella sp.	-	2,220	25±2	2	16	15	17	(Liang et al., 2013)
Chlorella vulgaris	2000 (mL/min)	18,500	25±1	1.48	40	26	26	(Griffiths et al., 2014)
Chlorella vulgaris	-	5,328	25±2	0.4	3	15	6	(Matos et al., 2014)
Chlorella sp.	1 (cm/s)	10,000	35±0.5	1.18	37	89	45	This work
Chlorella sp.	0.5 (cm/s)	30,000	30±0.5	1.11	33	68	61	This work

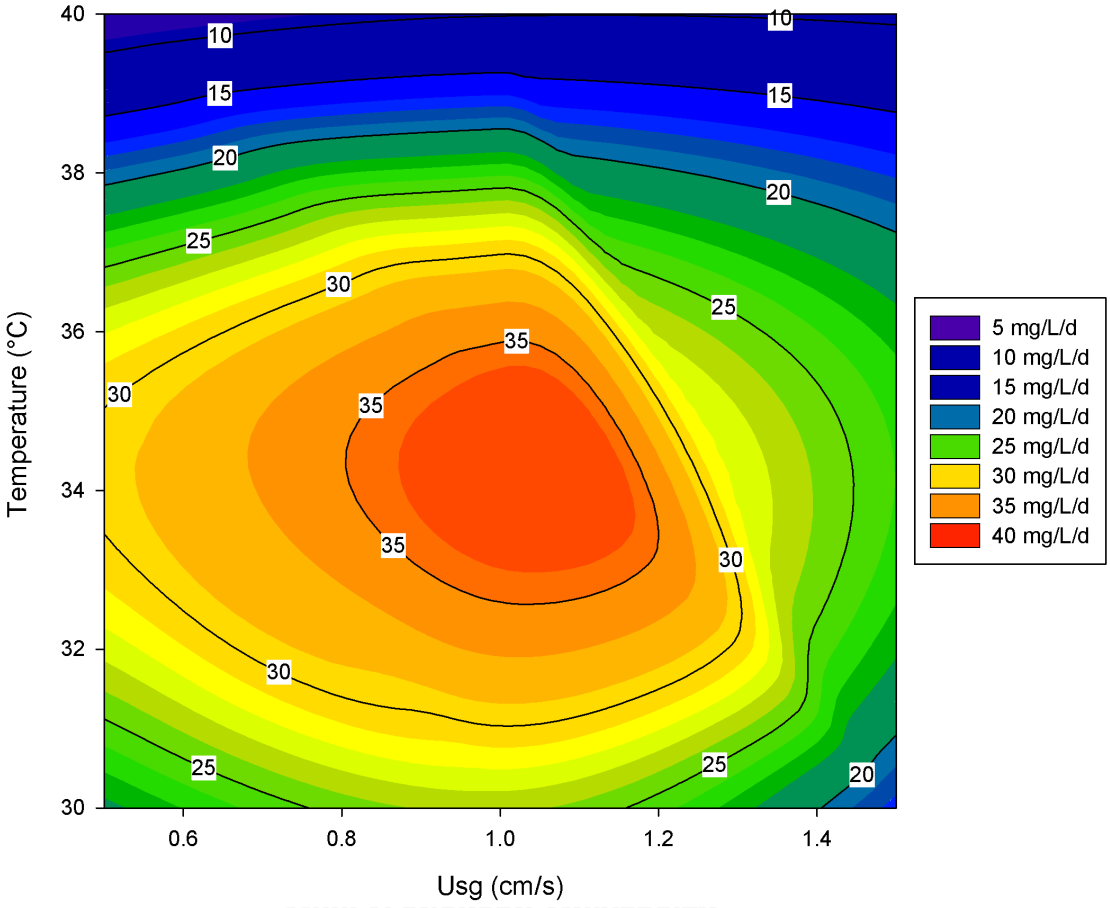

Figure 4.1 Lipid productivity (mg/L/d) of *Chlorella* sp. (Light intensity=10 kLux)

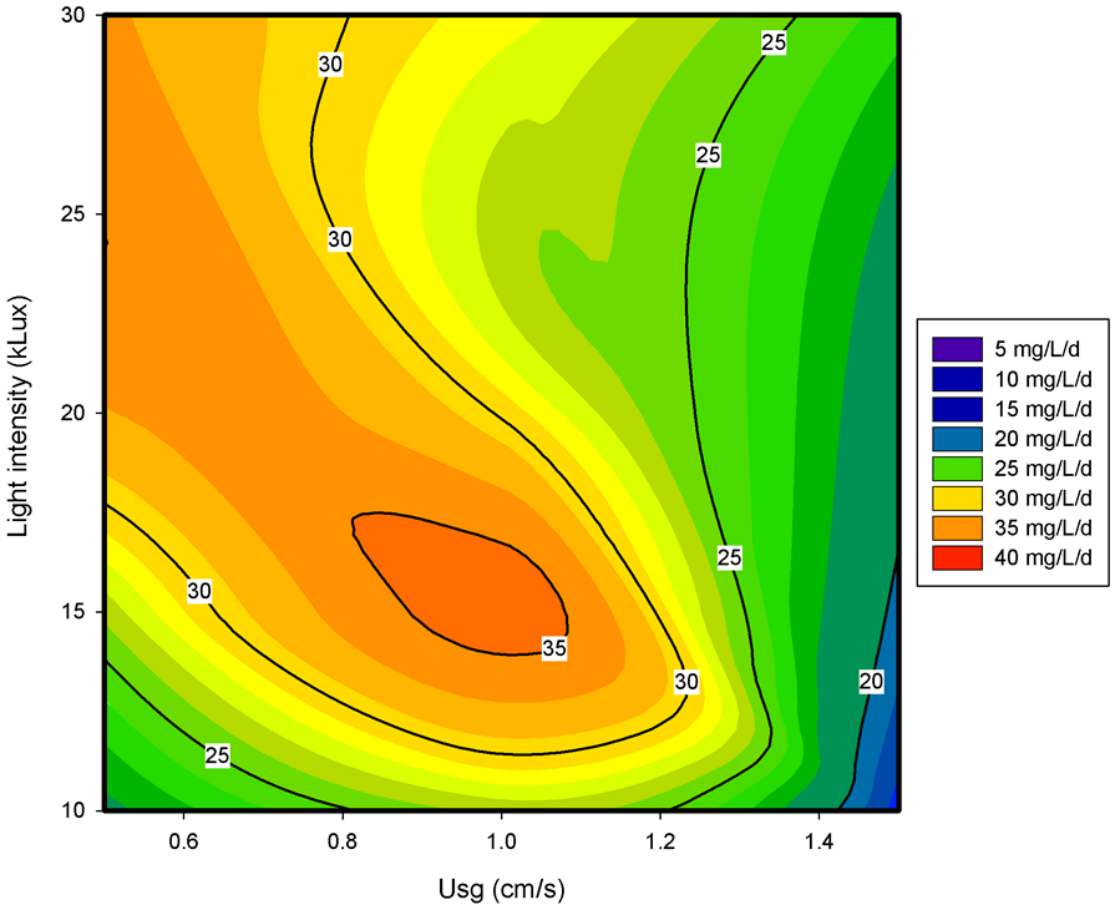

Figure 4.2 Lipid productivity (mg/L/d) of *Chlorella* sp. (Temperature=30 °C)

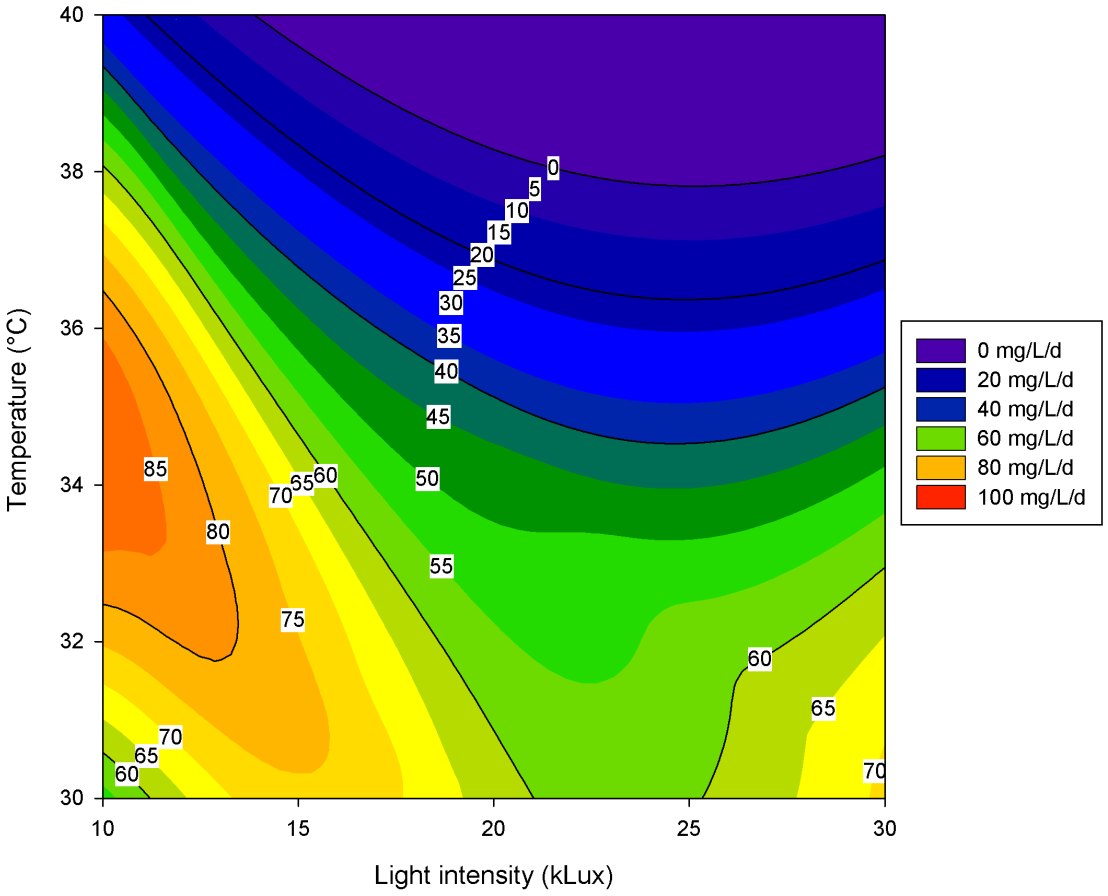

Figure 4.3 Protein productivity (mg/L/d) of *Chlorella* sp. (Usg=1 cm/s)

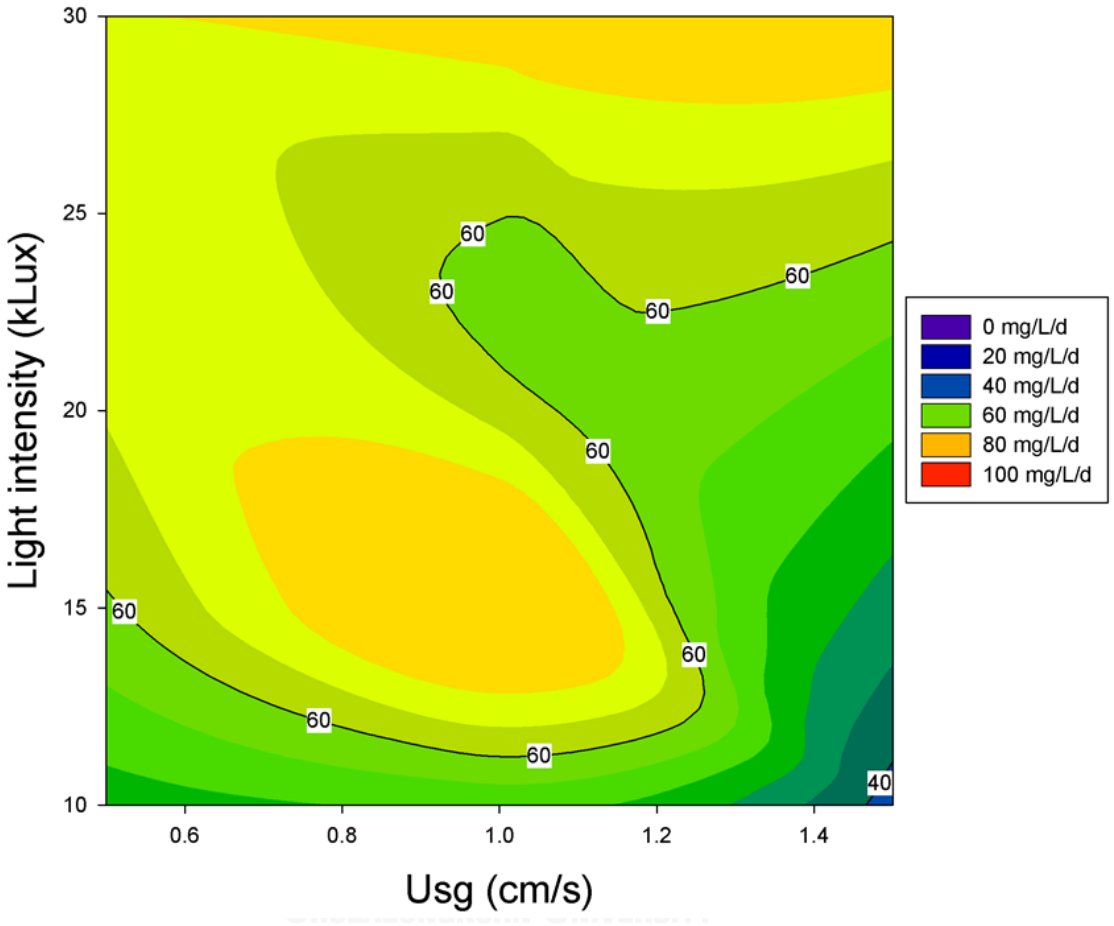

Figure 4.4 Protein productivity (mg/L/d) of *Chlorella* sp. (Temperature=30 °C)

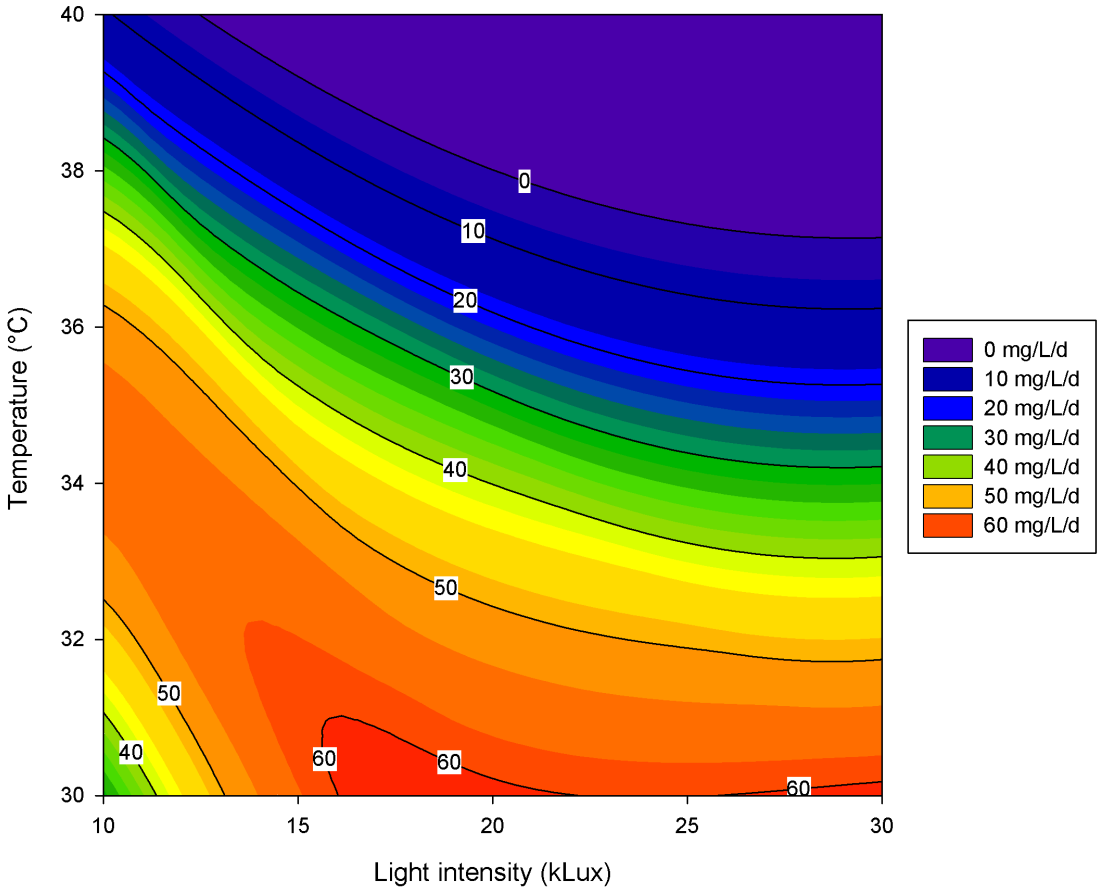

Figure 4.5 Carbohydrate productivity (mg/L/d) of *Chlorella* sp. (Usg=0.5 cm/s)

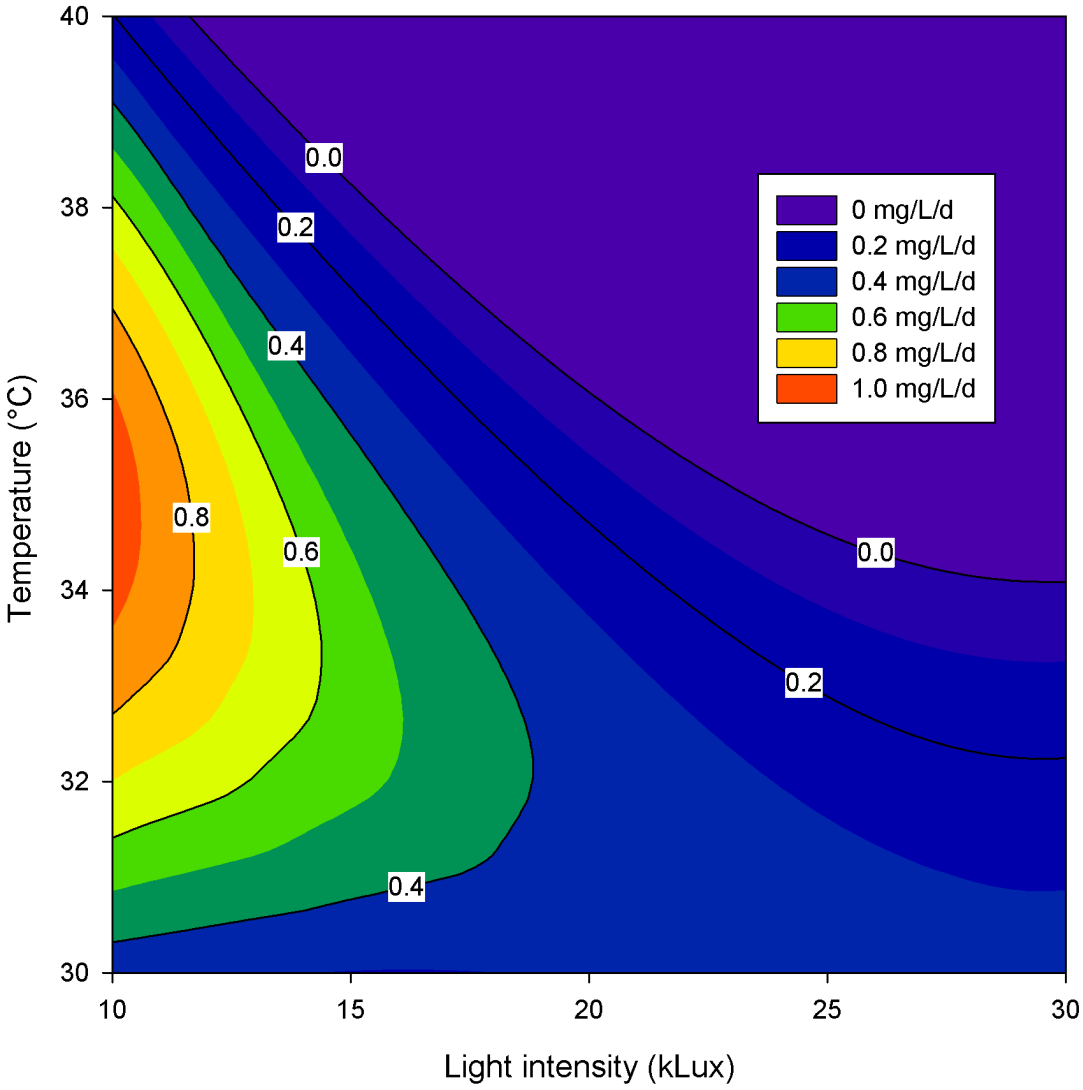

Figure 4.6 Lutein productivity (mg/L/d) of *Chlorella* sp. (Usg = 1 cm/s)

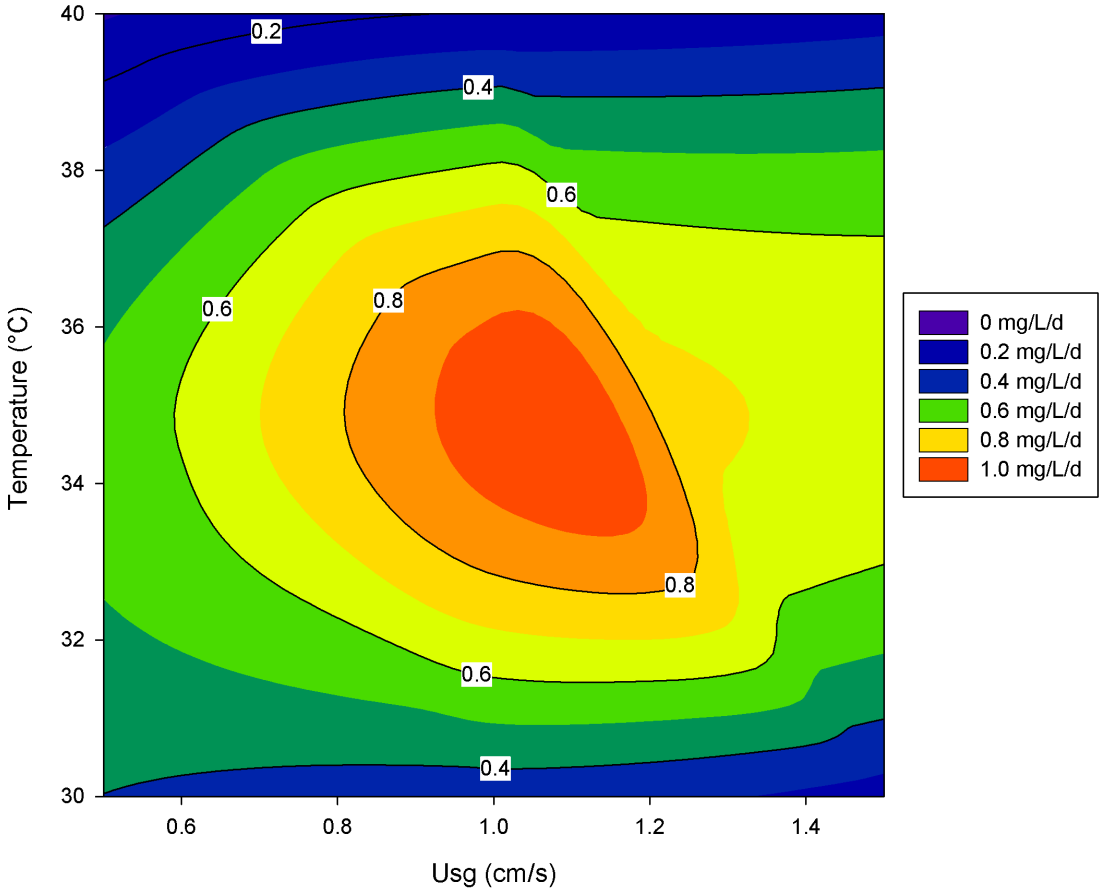

Figure 4.7 Lutein productivity (mg/L/d) of *Chlorella* sp. (Light intensity = 10 kLux)

Chapter V

Integrated Multi-trophic Recirculating Aquaculture System for Nile tilapia (*Oreochlomis niloticus*)

5.1 Introduction

Nile tilapia (*Oreochromis niloticus*) is a popular aquatic animal (Coward & Bromage, 2000, El-Sayed, 2006) due to their rapid growth rate (Hassanien *et al.*, 2004) especially male tilapia (Toguyeni et al., 2002), and generally high resistance to disease (Ardjosoediro & Ramnarine, 2002). Moreover, they also require relatively low oxygen for survival (El-Sayed & Kawanna, 2004, Ross, 2000, Siddiqui et al., 1989) and a natural surface oxygen transfer is generally adequate for their effective growth. This excludes the need for surface aeration which is a major electricity cost of the system. In 2009 alone, Thailand produced as much as 200,000 tons of tilapia production (Ferreira et al., 2014) which emphasizes the significance of this aquatic culture in the current human beings' food chain. However, typical culture practice for Nile tilapia in Thailand still does not incorporate the concept of integrated multi-trophic aquaculture system (IMTA) which renders the culture system susceptible to several environmental disturbances such as water quality, natural drought, etc. Moreover, Nile tilapia excretes wastes in the form of nitrogen and phosphorus compounds if not being treated properly can exert negative effects for the environment (Körner *et al.*, 2001, Lin *et al.*, 2001, Thanakitpairin et al., 2014, Thompson *et al.*, 2002). There is a therefore a clear need to develop the integrated multi-trophic recirculating aquaculture system (IMRAS) prototype as an integrated closed loop system for Nile tilapia-plankton-aquatic plants cultivation in Thailand to ensure future success of the system and to help guarantee the security of the food supply for the fast increasing global population. In this work, several aquatic plants, i.e. *Egeria densa*, *Ceratophyllum demersum*, *Hygrophila difformis*, *Vallisneria spiralis* and *Vallisneria Americana* were selected and cultured in the treatment tank to examine their effectiveness in the treatment of the nutrient rich wastewater from the fish tank. Moreover, the economics of the culture system was also enhanced by

introducing the combination of phytoplankton or microalgae (*Chlorella* sp.) cultivation and zooplankton (*Moina macrocopa*) could be further used as a high protein and nutrient feed for aquaculture hatcheries (Alam et al., 1993, Ingram, 2009, Peña-Aguado et al., 2009). By providing a proper balance between these various species, this RAS shares the common important concept with the integrated multi-trophic system where the synergistic relationship between the living organisms helps promote the sustainability and the economics of the whole system. The symbiotic mechanism of the various organisms in this IMRAS Nile tilapia culture was investigated.

5.2 Growth of Nile tilapia

The Integrated multi-trophic recirculating aquaculture system (IMRAS) was operated without replacing the water (fresh clean water was regularly added to replenish water lost by evaporation) for 336 days or three fish crops and the growth of the fish was demonstrated in Figure 5.1. The results indicate that the fish in the low density system (20 fish/m^3) grew at a faster rate when compared with those from the higher fish densities (i.e. 25 and 50 fish/m^3). However, the system with the fish density of 50 fish/m^3 provided the highest total productivity (wet-weight) of 11 ± 1 kg fish/m^3 whereas the densities of 20 and 25 fish/m^3 could only produce the total wet-weight of 6.8 ± 0.3 and 5.3 ± 0.5 kg fish/m^3, respectively. The average wet-weight of Nile tilapia at density of 50 fish/m^3 increased from 2.4 ± 0.6 g/fish to 240 ± 16 g/fish within 112 days of growth which corresponds to the average daily weight gain of 2.1 ± 0.1 g/fish-day. Feed conversion ratio (FCR) and survival rate were 1.5 ± 0.2 and 91± 1 %, respectively. These are reasonably good when compared with the results obtained from the other density condition and therefore the density of 50 fish/m^3 was selected for further investigation. The summary of growth characteristics (weight gain, daily weight gain, FCR, and survival rate) is given in Table 5.1. Note that the growth characteristics obtained from this system is comparable to those from other reported systems, e.g. the daily weight gain from the "recirculating greenwater system" was 1.75 g/fish-day (Al-Hafedh & Alam, 2005) and from the "cage culture system" was 1.15 ± 0.02 g/fish-day (Gibtan et al., 2008).

5.3 Growth of *Chlorella* sp. and *Moina macrocopa*

Chlorella sp. could grow reasonably well from 0.01 to 0.2 g/L (Figure 5.3,◇) within 4 days considered that the system was operated under uncontrolled environmental parameters (light intensity and temperature). Figure 5.2 illustrates that such growth could be significantly enhanced if the cultivation parameters, e.g. temperature, light intensity and exposure period, could be well controlled. With the outdoor *Chlorella* sp. culture as a feed, *Moina macrocopa* could grow well and the density increased from 0.1 to 0.4 g/L within 4-5 days (Figure 5.3,△) which was slightly better than the value reported in previous literature (Martínez-Jerónimo & Gutierrez-Valdivia, 1991).

The results demonstrate that it was possible to enhance the economics of the system by introducing proper bio-components in the food chain. In this case, a high value animal feed *Moina macrocopa* (2-3 $/kg) was introduced to convert and upgrade the low value *Chlorella* sp. biomass which was again fed on NH_3 excreted from the fish culture. It is interesting to observe that, during the growth of *Chlorella* sp., NH_3 was being used for growth and the concentration of NH_3 dropped significantly. The level of NH_3 bounced back again (Figure 5.3,○) as *Moina macrocopa* grew as it also excreted NH_3 during its growth stage.

5.4 Growth of aquatic plants

Figure 5.4 illustrates the wet-weights of the five aquatic plants which were harvested every 14 days. The different aquatic plants grew at different rates but the productivities of all aquatic plants followed the same pattern. Most aquatic plants grew at a relatively slow rate at the beginning which was due to the limited nitrogen source. In other words, the initial concentrations of nitrogen and phosphorus levels from the fish tank that flew into the aquatic plants tank were inadequate for growth (as the fish was still small). The growth rate increased considerably particularly for *Egeria densa* and *Ceratophyllum demersum* during the first 42 days implying not only that there was a more abundant nitrogen/phosphorus compounds due to the accumulation of the uneaten or remaining feed, but also from the acquisition of the

aquatic plants to the environment of the tank. The other aquatic plants, i.e. *Hygrophila difformis, Vallisneria spiralis, and Vallisneria Americana*, also grew at a faster rate but the changes in the growth rate were not as obvious when compared to the two species mentioned above. The total fresh weight of all aquatic plants could be ordered from high to low as follows: *Egeria densa* (14.9±0.7 kg), *Ceratophyllum demersum* (13.2±0.5 kg), *Vallisneria Americana* (3.87±0.09 kg), *Vallisneria spiralis* (3.67±0.03 kg), and *Hygrophila difformis* (1.74±0.06 kg).

It is noted that aquatic plants directly assimilated nitrogen and phosphorus into the biomass. However, the analysis of N balance in the following section shows that this nitrogen assimilation only accounted for a small fraction of nitrogen input (in animal feed), and most of the nitrogen was lost from other unknown mechanism which, in this case, was believed to be the conversion of NH_3 and NH_4^+ to NO_3^- via nitrifying bacteria and perhaps also via the denitrifying activities as the level of oxygen under the water level in the tank could well exhibit anaerobic condition. These groups of bacteria were generally found in the sediment of the culture tank (Cossu et al., 2001, Zhao et al., 2014).

Aquatic plants were also reported to have beneficial effect as they acted like a filter for the suspended sediment (Ostroumov, 1998). This helped enhance the level of dissolved oxygen (DO) in the fish tank, as this sediment is usually organic matters which could undergo aerobic decomposition in the fish tank. Preventing this organic decomposition therefore eliminated the unnecessary oxygen uptake in the fish tank resulting in a better control of DO level in the cultivating system (Cheng & Stomp, 2009, Kapuscinski et al., 2014).

The findings from this section suggest that the selection of the aquatic plants species used in the treatment tank should be carefully considered to ensure high treatment efficiency and also a reasonably level of economic benefit. The rapid growth plants must be used to provide a reliable water treatment/filtering capacity whereas the slow growth plants should also be provided as they are usually of high value and could enhance the feasibility of the system.

5.5 Water quality

Figure 5.5 illustrates nitrogen and phosphorous compounds profiles in the fish and aquatic plants tanks where nitrogen (NH_3, NH_4^+, NO_2^- and NO_3^-) and phosphorous (PO_4^{3-}) levels in both tanks continuously increased during the first 50 days and remained constant until the end of experiment. Maximum nitrogen and phosphorous concentrations in the fish tank were 0.38±0.02, 0.57±0.02, 55±2 mgN/L and 0.32±0.03 mgP/L, for ammonia, nitrite, nitrate and phosphate, respectively. These levels of nitrogen compounds were still lower than the dangerous level for Nile tilapia (dangerous level indicated by the dash line in Figure 5.5 as suggested by (Hart et al., 1993, Liao & Mayo, 1972, Masser et al., 1999), but still higher than those in the aquatic plants tank where the corresponding concentrations were reduced to 0.28±0.02, 0.33±0.02, 38±2 mgN/L and 0.20±0.02 mgP/L, respectively. This indicates that the water in the treatment system could be self-cleaned by the provided concocted ecosystem. It is noted that the level of ammonia, nitrite, nitrate and phosphate at the end of the control system were 0.52±0.04, 1.20±0.04, 135±11 mgN/L and 2.45±0.04 mgP/L which were relatively high, indicating inadequate treatment capacity in such system.

Dissolved oxygen (DO) gently decreased both in the fish and aquatic plants tank. The initial DO concentrations in both tanks were 6.45 and 5.97 mg/L and the final concentrations were 5.55 and 4.65 mg/L, respectively (Figure 5.6 A). In the fish tank, the reduction in DO would be due to a greater need for oxygen from the larger fish (Dam & Pauly, 1995). On the other hand, despite oxygen generated from photosynthesis, more oxygen was also required in the aquatic plants tank due primarily to the decomposition of uneaten feed and fish feces and nitrogen compounds through nitrification reaction. DO in the *Chlorella* sp. and *Moina macrocopa* tanks remained mostly unchanged (data were not shown) indicating that the activities of the tank could be maintained regardless of the conditions in the other tanks.

Figure 5.6 also demonstrates the variation in temperature, alkalinity and pH in the system. Due to a large quantity of water, the uncontrolled system temperature was in the range of 27-32°C with an average of 28-29°C which was considered within the

optimum range (25-30°C) for Nile tilapia (Balarin & Haller, 1982, Chervinski, 1982, Philippart & Ruwet, 1982). Figure 5.6 C demonstrates that alkalinity dropped with time which was potentially due to the activity of nitrifying bacteria and some other algae that might grow in the system. However, Hart et al. (1993) suggested that the alkalinity for aquatic animals should be maintained above 100 mg-$CaCO_3$/L, therefore, $NaHCO_3$ was added to stabilize the level of alkalinity above this level. The addition of $NaHCO_3$ could, also stabilize the pH value in the system, and Figure 5.6 D illustrates that pH (at 2 p.m.) could be naturally controlled within the range of 6-8.5 which was save for the living organisms involved in the ecology of this system.

5.6 Nitrogen and phosphorus mass balances

Figure 5.7 is the summary of the flow of nitrogen compounds within IMRAS. In this experiment, total nitrogen input (790.63 g) came from the use of fish feed throughout the 112 days of each crop. A large quantity of nitrogen (301.13 g N or 38.09% of the total nitrogen input) could be converted to Nile tilapia. This level is more or less within the range reported elsewhere (Rafiee & Saad, 2005, Trang & Brix, 2014). The remaining nitrogen was converted to: *Chlorella* sp. (7.64 g or 0.97%), *Moina macrocopa* (1.88 g or 0.24%), and aquatic plants (75.29 g or 9.52%). Some nitrogen, e.g. ammonia, nitrite, nitrate (about 91.08 g or 11.52%) was still dissolved in the water at the end of the experiment. Some of nitrogen (48.31 g or 6.11%) might still be adsorbed in the soil while some of nitrogen was not measured directly but calculated as the unaccounted nitrogen. As much as 265.30 g (33.56%) could undergo the decomposition reaction carried out by denitrifying bacteria resided within the eco-system such as at the soil sediment in the aquatic plants tank.

Similarly, Figure 5.8 displays the flow of phosphorus within IMRAS where the total phosphorus entering the system was 172.01 g (mostly in the fish feed). The amounts of phosphorus converted to Nile tilapia, *Chlorella* sp., *Moina macrocopa* and aquatic plants were 52.75 g (30.67%), 1.20 g (0.70%), 0.22 g (0.13%), and 19.61 g (11.40%), respectively (Figure 5.8). Again, some phosphorus was still soluble in the water at the end of the experiment and this accounted for about 2.65% (or 4.55 g) of the total phosphorus input. As much as 93.67 g or 54.46% of phosphorus could not be

accounted for by the measurement employed in this work. This phosphorus was anticipated to remain partially in the soil matrix and some could be assimilated to the microorganisms cultivated within the system.

Figures 5.7 and 5.8 summarize that nitrogen and phosphorous of 1,311 ± 26 $mgN/m^3/d$ and 230 ± 21 $mgP/m^3/d$ were converted to Nile tilapia mass with an initial fresh weight of 2.4 ± 0.6 g/fish and with a density of 50 $fish/m^3$. This was from the cultivation period of 112 days where the final fresh weight was 240 ± 16 g/fish as shown in Figure 5.9. Phytoplankton and zooplankton could only convert a small fraction of nitrogen and phosphorous to biomass, i.e. at about 41 ± 7 $mgN/m^3/d$ and 6 ± 1 $mgP/m^3/d$. Nitrogen and phosphorous of 328 ± 80 $mgN/m^3/d$ and 85 ± 16 $mgP/m^3/d$, respectively, were converted into all aquatic plants (*Egeria densa*, *Ceratophyllum demersum*, *Vallisneria Americana*, *Vallisneria spiralis*, and *Hygrophila difformis*). Nitrogen and phosphorous amounts of 397 ± 37 $mgN/m^3/d$ and 20 ± 3 $mgP/m^3/d$, respectively, were dissolved in the water, while nitrogen of 1,155 ± 114 $mgN/m^3/d$ and phosphorous of 408 ± 26 $mgP/m^3/d$ could not be utilized. This finding suggested that the remaining nitrogen and phosphorous could still be utilized by aquatic plants provided that there are enough area for the plants to grow. A rough linear estimate recommended that the area for the aquatic plants should increase 4-5 times to accommodate the amount of the remaining nitrogen and phosphorus.

5.7 Conclusions

This work demonstrates the success of the implementation of the close loop aquacultural system where the treatment tank is introduced. The treatment tank completes the ecology of the fish culture by providing proper conversion of the wastes generated from the fish by turning them into other valuable products, in the case, phytoplankton, zooplankton, and aquatic plants. Not only that this system benefits from these added value by-products, but it also enables the recirculation of the culture water, enhancing the reliability of the water management within the system. With the treatment tank, the water quality; ammonia, nitrite nitrate, phosphate, pH and DO could be well controlled at safe level through the cultivation duration of 112 days, and the observed fish productivity was reasonably high.

Table 5.1 Growth characteristics of Nile tilapia in the treatment system (mean value)

Growth parameter	Fish stocking (Fish/m^3)		
	20	25	50
Initial mean weight (g)	2.6±0.5	2.6±0.6	2.4±0.6
Final mean weight (g)	344±69	252±37	240±16
Weight gain (g)	342±15	249±3	237±16
Daily weight gain (g/d)	3.1±0.1	2.23±0.03	2.1±0.1
Feed Conversion Ratio; FCR	1.36±0.06	1.2±0.1	1.5±0.2
Survival rate (%)	95	86±3	91±1
Productivity (kg/m^3)	6.8±0.3	5.3±0.5	11±1

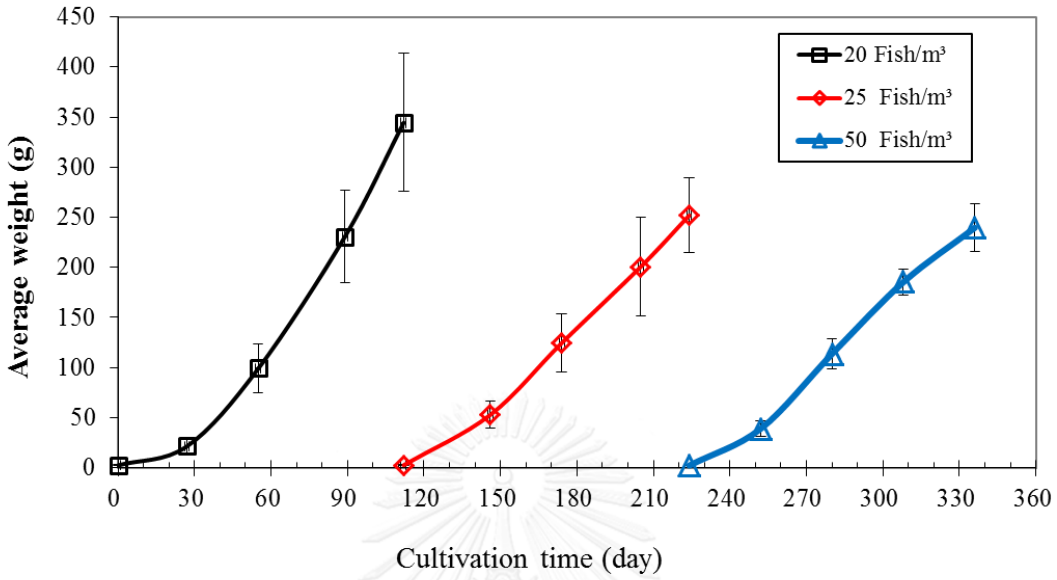

Figure 5.1 Growth curve of Nile tilapia

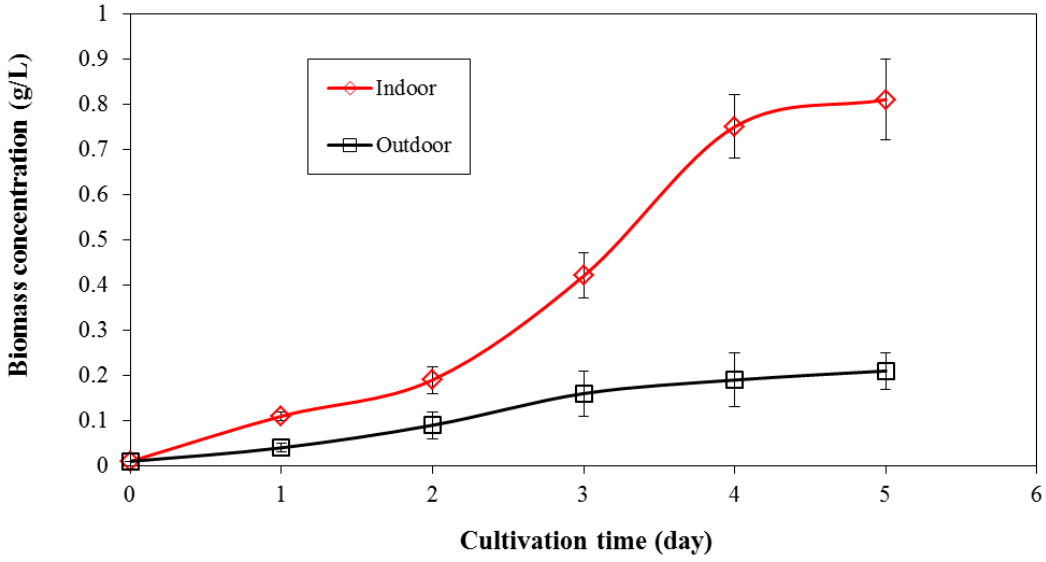

Figure 5.2 Growth curve of *Chlorella* sp. in Indoor (temp: 30°C, light intensity: 10,000 LUX, light exposure period: 24 hours) and Outdoor cultivations (uncontrolled environmental parameters)

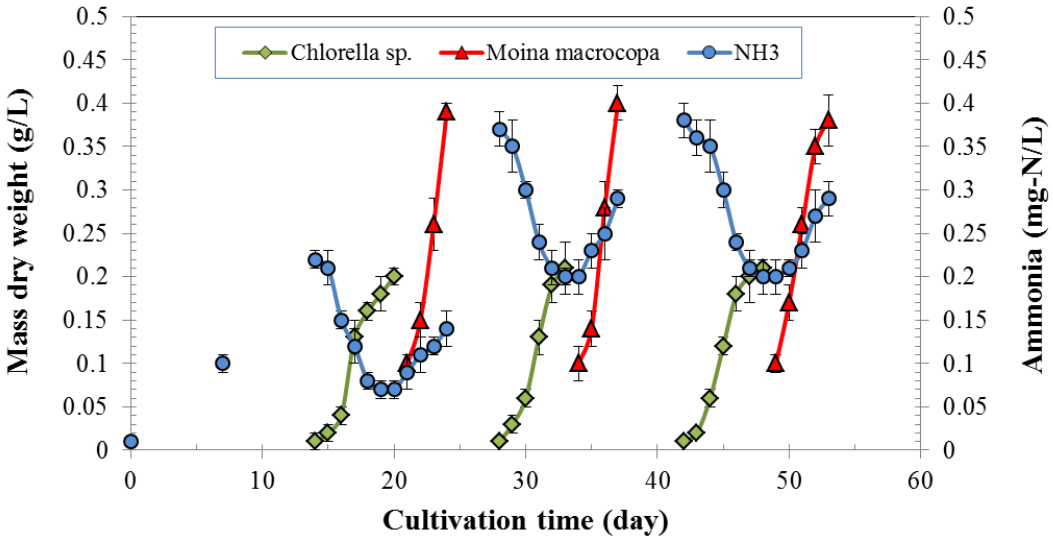

Figure 5.3 Growth curve of *Chlorella* sp. (◇) and *Moina macrocopa* (△) and NH_3 concentration profile (○)

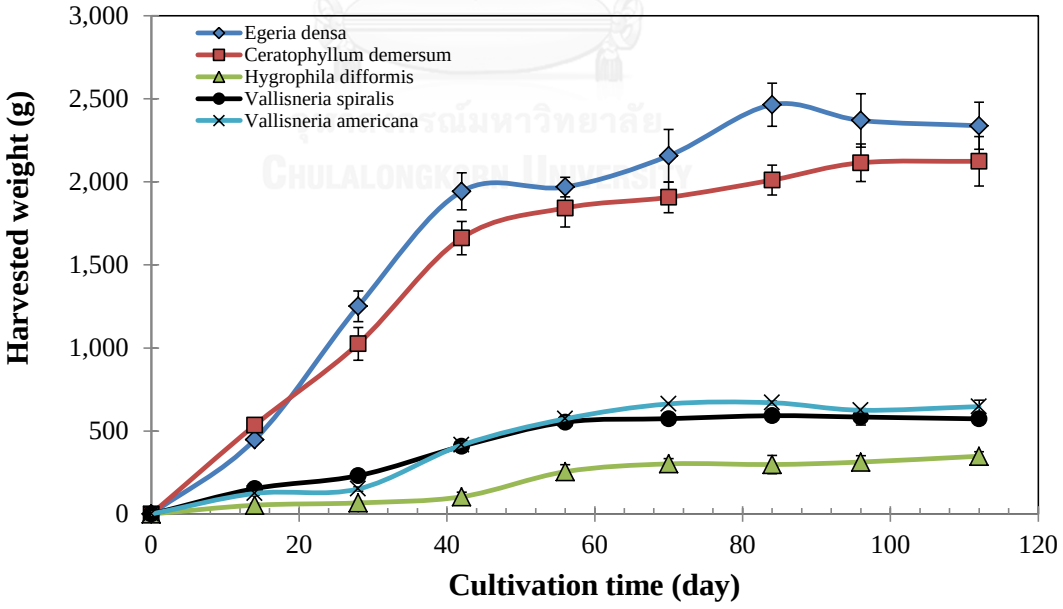

Figure 5.4 Average harvested (14 days harvesting interval weight of aquatic plants)

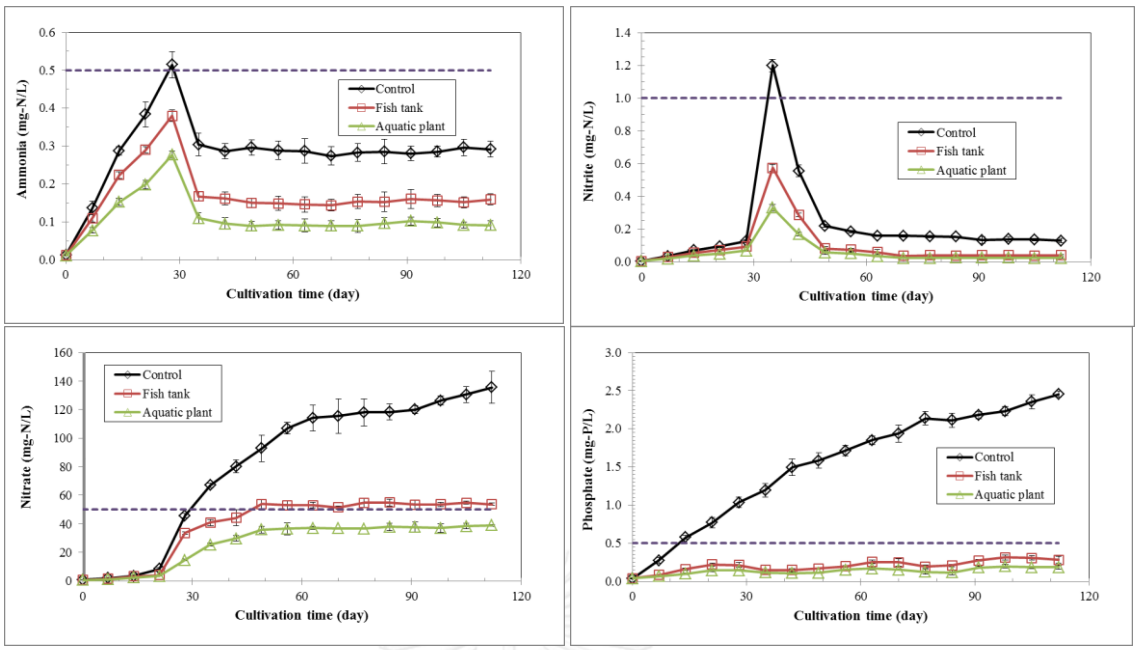

Figure 5.5 Concentration of ammonia, nitrite, nitrate, and phosphate in fish and aquatic plants tank

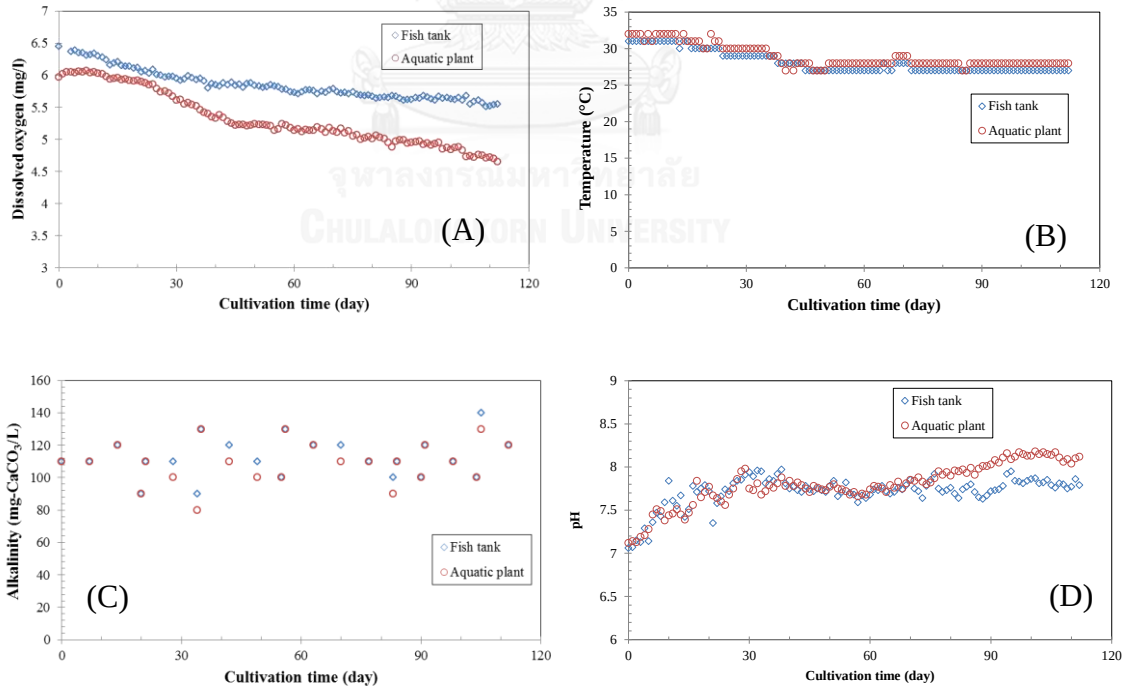

Figure 5.6 Water qualities in fish and aquatic plants tank: Dissolved oxygen (A); temperature (B); alkalinity (C) and pH (D)

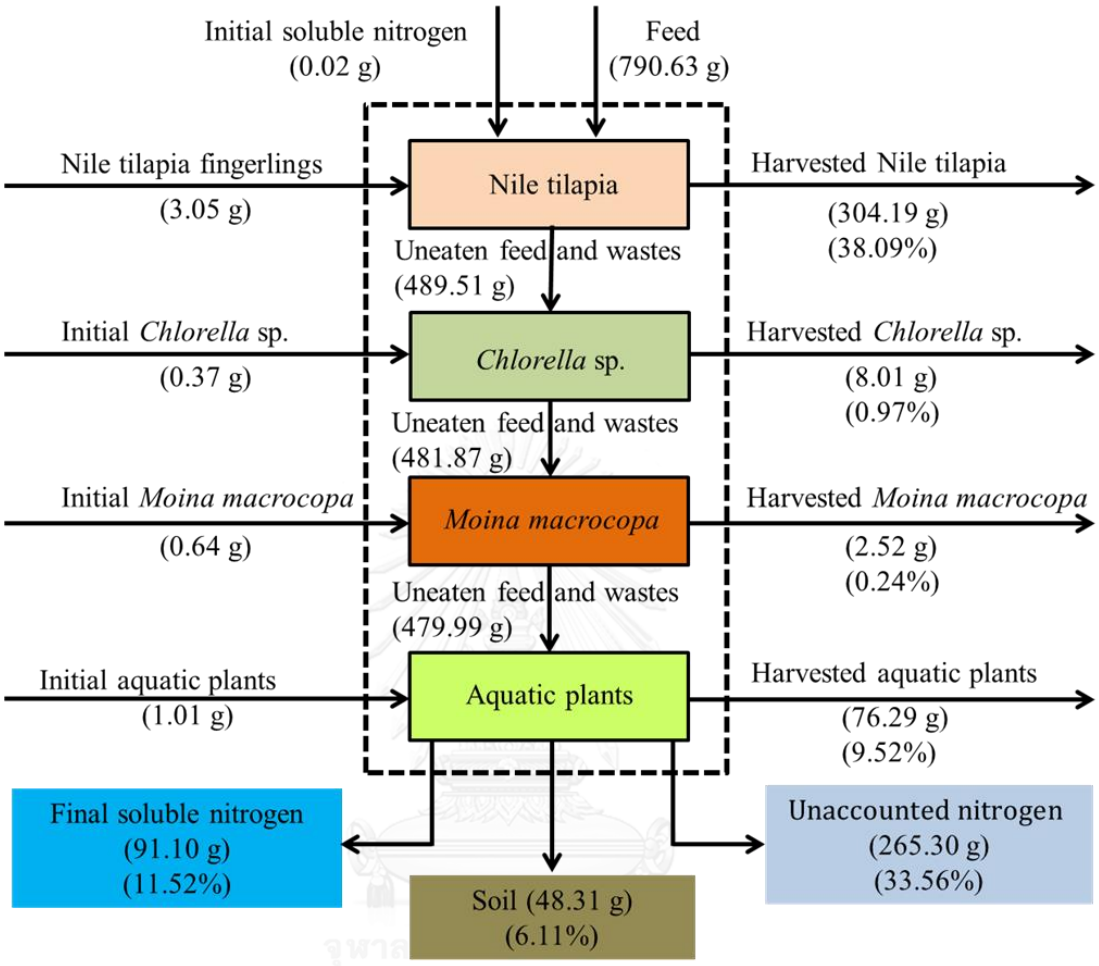

Figure 5.7 Nitrogen balance of IMRAS

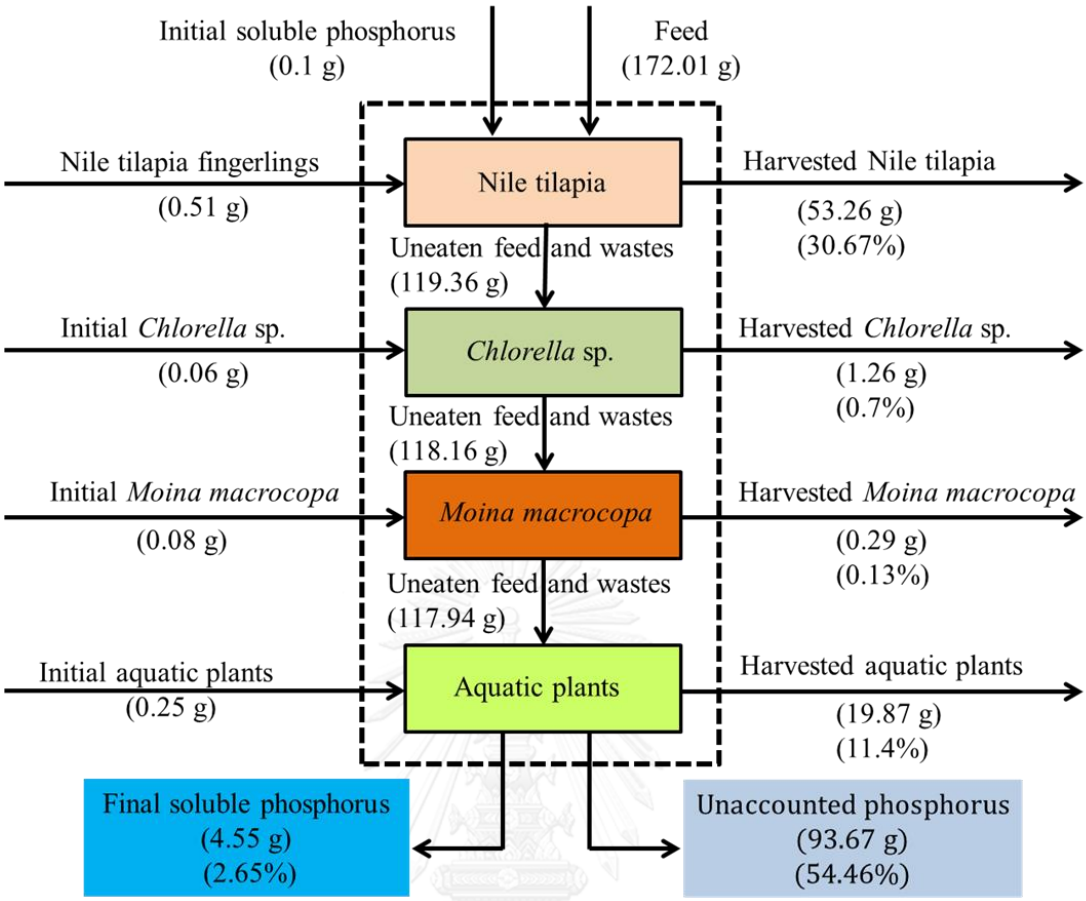

Figure 5.8 Phosphorous balance of IMRAS

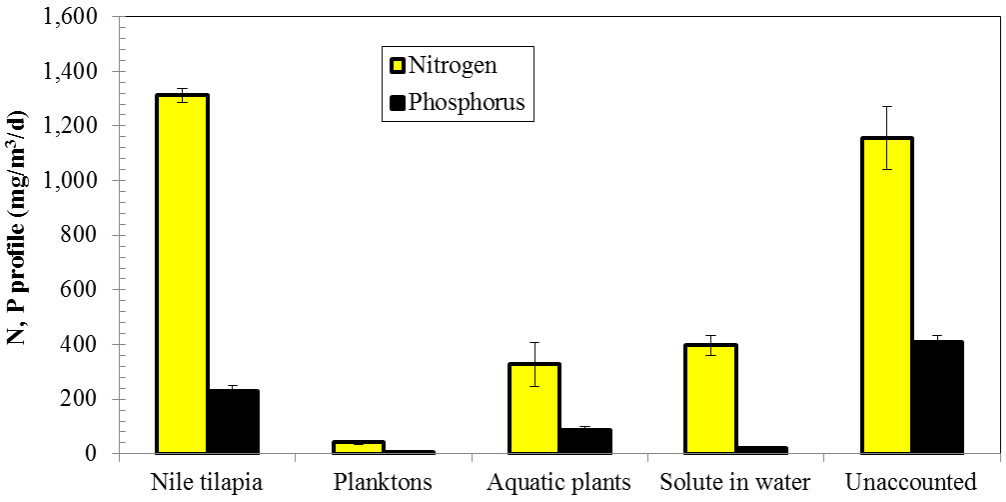

Figure 5.9 Nitrogen and phosphorous final profile

Chapter VI
Conclusions and contributions

6.1 Biochemical composition productivity of *Chlorella* sp.

The biochemical composition (lipid, protein, carbohydrate, and lutein) accumulated during the cultivation of *Chlorella* sp. could be adjusted, although slightly in some cases, by manipulating typical culture conditions at its most appropriate level, such as temperature, light intensity and aeration velocity. The selection of operating conditions therefore needs to be carefully considered to ensure that the final quality of the product could be achieved. The various cultivating conditions for the different purposes are summarized below.

6.1.1 Lipid

The cultivation of *Chlorella* sp. at light intensity, temperature, and aeration rate of approximately 10 kLux, 35°C and 1 cm/s, respectively, can produce the highest lipid productivity (37 mg/L/d). Lipid productivity decreased when departing culture condition from the optimal condition, especially at high temperature, e.g. 38-40°C. The culture might require a higher light intensity to maintain the lipid productivity at this high level if the temperature or mixing deviated from this optimal range. For instance, the light intensity at 15 kLux was required when *Chlorella* sp. was cultivated at 30°C, or at 25 kLux when the aeration rate was 0.5 cm/s.

6.1.2 Protein

The highest protein productivity (89 mg/L/d) was achieved at light intensity, temperature, and aeration rate of 10 kLux, 35°C, and 1 cm/s, respectively. Protein productivity decreased significantly to 53 and 29 mg/L/d when temperature changed to 30 and 40°C, respectively, due to the poor growth rate. At cultivation temperature of about 30°C, high protein accumulation might be achieved by providing high light intensity and aeration rate. For example, increasing light intensity and aeration

velocity from 15 to 30 kLux and 1 to 1.5 cm/s could notably increase the protein productivity from 53 to 74 mg/L/d.

6.1.3 Carbohydrate

The highest carbohydrate productivity of 60 mg/L/d were obtained at two ranges of light intensity, i.e. at between 16 and 22 kLux, and at 30 kLux, both at temperature of 30°C and aeration velocity of 0.5 cm/s. Carbohydrate productivity decreased significantly at other operating conditions.

6.1.4 Lutein

The highest lutein productivity of 0.9 mg/L/d occurred at light intensity, temperature and aeration velocity of 10 kLux, 35°C and 1 cm/s. Lutein productivity seemed to decrease with increasing light intensity to greater than 10 kLux. To keep high lutein productivity, temperature had to be controlled within the range of 33-37°C, aeration rate of 0.8-1.2 cm/s, and with light intensity of 10 kLux. At these conditions, lutein productivity was in the range of 0.8-0.9 mg/L/d (270 mg/m^2/d) which was about 16 times higher than that from marigold.

6.2 Integrated Multi-trophic Recirculating Aquaculture System (IMRAS)

6.2.1 Growth of Nile tilapia in IMRAS

The system with the fish (nile tilapia) density of 50 fish/m^3 provided the highest total productivity (wet-weight) of 11 ± 1 kg fish/m^3 whereas the densities of 20 and 25 fish/m^3 could produce the total wet-weight of 6.8 ± 0.3 and 5.3 ± 0.5 kg fish/ m^3, respectively. The average wet-weight of Nile tilapia at density of 50 fish/m^3 increased from 2.4 ± 0.6 g/fish to 240 ± 16 g/fish within 112 days of growth which corresponded to the average daily weight gain of 2.1 ± 0.1 g/fish-day. Feed conversion ratio (FCR) and survival rate were 1.5 ± 0.2 and 91± 1 %, respectively.

6.2.2 Growth of *Chlorella* sp. and *Moina macrocopa* in IMRAS

Chlorella sp. could grow reasonably well from 0.01 to 0.2 g/L within 4 days when operated under uncontrolled environmental parameters (light intensity and temperature). This growth could be significantly enhanced if the cultivation parameters, e.g. temperature, light intensity and exposure period, could be well controlled. With the outdoor *Chlorella* culture as a feed, *Moina macrocopa* could grow well and the density increased from 0.1 to 0.4 g/L within 4-5 days.

6.2.3 Growth of aquatic plants in IMRAS

The different aquatic plants grew at different rates but the productivities of all aquatic plants followed the same pattern. The growth rate of aquatic plants increased considerably particularly for *Egeria densa* and *Ceratophyllum demersum* during the first 42 days. *Hygrophila difformis, Vallisneria spiralis, and Vallisneria Americana*, also grew at a faster rate but the changes in the growth rate were not as obvious when compared with *Egeria densa* and *Ceratophyllum demersum*. The total fresh weight could be ordered from high to low as follows: *Egeria densa* (14.9±0.7 kg), *Ceratophyllum demersum* (13.2±0.5 kg), *Vallisneria Americana* (3.87±0.09 kg), *Vallisneria spiralis* (3.67±0.03 kg), and *Hygrophila difformis* (1.74±0.06 kg).

6.2.4 Water quality

Maximum nitrogen (NH_3/NH_4^+, NO_2^- and NO_3^-) and phosphorous (PO_4^{3-}) concentrations in the fish tank in IMRAS were 0.38±0.02, 0.57±0.02, 55±2 mgN/L and 0.32±0.03 mgP/L, for ammonia, nitrite, nitrate and phosphate, respectively. These levels of nitrogen compounds were still lower than the dangerous level for Nile tilapia, but still higher than those in the aquatic plants tank where the corresponding concentrations were reduced to 0.28±0.02, 0.33±0.02, 38±2 mgN/L and 0.20±0.02 mgP/L, respectively. This indicates that the water in the treatment system could be self-cleaned by the provided concocted ecosystem. It is noted that the level of ammonia, nitrite, nitrate and phosphate at the end of the control system (unintegrated,

stand-alone tank) were 0.52±0.04, 1.20±0.04, 135±11 mgN/L and 2.45±0.04 mgP/L which were relatively high, indicating inadequate treatment capacity in such system.

The initial dissolved oxygen (DO) concentrations in both fish and aquatic plants tanks were 6.45 and 5.97 mg/L and the final concentrations were 5.55 and 4.65 mg/L, respectively. Dissolved oxygen in the *Chlorella* sp. and *Moina macrocopa* tanks remained mostly unchanged. The temperature in this system was in the range of 27-32°C with an average of 28-29°C which was considered within the optimum range (25-30°C) for Nile tilapia. The pH (at 2 pm) could be naturally controlled within the range of 6-8.5 which was safe for the living organisms involved in the ecology of this system.

6.2.5 Nitrogen and phosphorus mass balances

The total nitrogen input (790.63 g) came from the use of fish feed throughout the 112 days of cultivation. A large quantity of nitrogen (38.09% of the total nitrogen input) could be converted to Nile tilapia. The remaining nitrogen was converted to: *Chlorella* sp. (0.97%), *Moina macrocopa* (0.24%), and aquatic plants (9.52%). Some nitrogen, e.g. ammonia, nitrite, nitrate (11.52%) was still dissolved in the water at the end of the experiment. As much as 39.67% of nitrogen was not measured directly but calculated as the unaccounted nitrogen. Some of this nitrogen might still be adsorbed in the soil while some also could undergo the decomposition reaction carried out by denitrifying bacteria resided within the eco-system such as at the soil sediment in the aquatic plants tank.

Phosphorus entering the system was 172.01 g. The amounts of phosphorus converted to Nile tilapia, *Chlorella* sp., *Moina macrocopa* and aquatic plants were 30.67, 0.70, 0.22, and 11.40%, respectively. Some phosphorus was still soluble in the water at the end of the experiment and this accounted for about 2.65% of the total phosphorus input. As much as 54.46% of phosphorus could not be accounted for by the measurement employed in this work. This phosphorus was anticipated to remain partially in the soil matrix and some could be assimilated to the microorganisms cultivated within the system.

6.3 Contributions

The findings from this work can lead to two major contributions as described below:

- The biochemical composition productivity (lipid, protein, carbohydrate and lutein) of *Chlorella* sp. could be partially manipulated through the control of cultural conditions (light intensity, temperature, and aeration rate). Similar algorithm might also work for other algal species.

- The use of synthetic ecosystem such as multi-trophic culture can control the concentrations of ammonia, nitrite, nitrate, and phosphate in the system below dangerous level for Nile tilapia. This should well be applied for other aquaculture systems.

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APPENDIX

Effects of environmental conditions on nutritional productivity of *Chlorella* sp.

Effect of light intensity

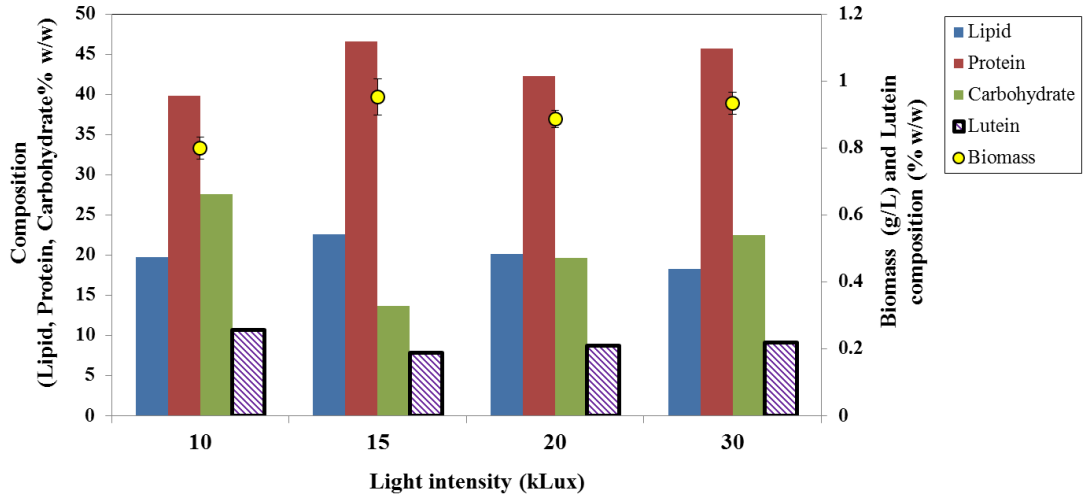

Figure A.1 Biochemical composition and biomass of *Chlorella* sp. at temperature and aeration rate of 30°C and 1 cm/s, respectively

Effects of temperature

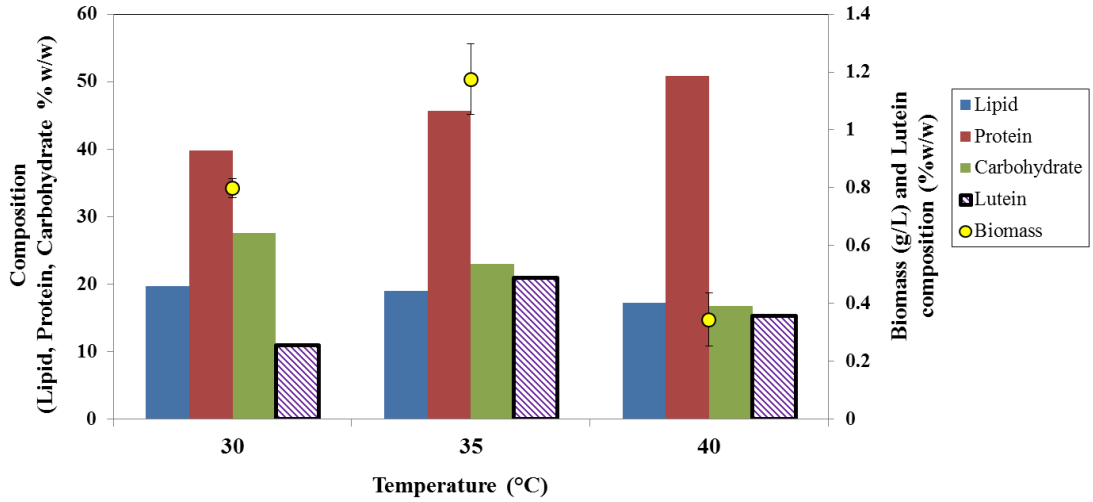

Figure A.2 Biochemical composition and biomass of *Chlorella* sp. at light intensity and aeration rate of 10 kLux and 1 cm/s, respectively

Effects of aeration rate

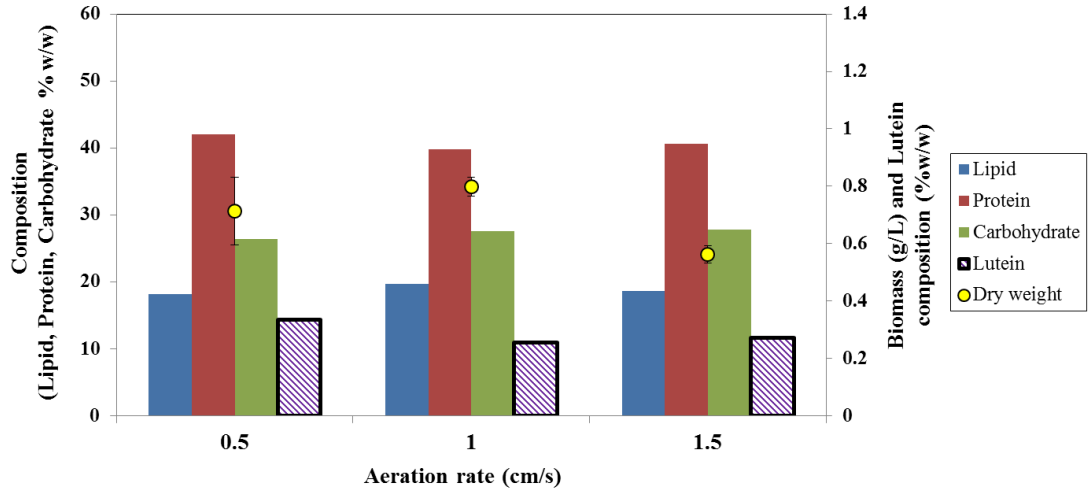

Figure A.3 Biochemical composition and biomass of *Chlorella* sp. at light intensity and temperature of 10 kLux and 30°C, respectively

VITA

Mr. Puchong Sri-uam was born on May 26th 1982 in Ratchaburi, Thailand. He finished his higher secondary course from Benjamarachutit Ratchaburi School in March 2001. After that, he studied in the Major of Chemical Engineering in Faculty of Engineering at King Mongkut's University of Technology North Bangkok. He continued his further study for Master's degree in Chemical Engineering at Chulalongkorn University in 2005. He participated in the Biochemical Engineering Research Group and achieved his Master's degree in March, 2007. He continued studying Ph.D. in Chemical Engineering in Environmental Chemical Engineering Research Group since 2009. He was granted Dutsadi Phiphat Scholarship of Chulalongkorn University and took this opportunity to be an exchange student at Kyoto University, Kyoto, Japan for 5 months. He achieved his Ph.D. degree in 2015.