ผลจากการเติมสารอินทรีย์คาร์บอนต่อการเกิดตะกอนจุลินทรีย์และคุณภาพน้ำ
ในระบบการเลี้ยงสัตว์น้ำแบบปิด

นางสาววรรัตน์ วณิชชานัย

EFFECTS OF ORGANIC CARBON ADDITION ON MICROBIAL FLOC
FORMATION AND WATER QUALITY IN CLOSED AQUACULTURE SYSTEM

Miss. Worarat Vanitchanai

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
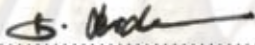Department of Chemical Engineering
Faculty of Engineering
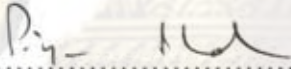Chulalongkorn University
Academic Year 2009

Thesis Title   EFFECTS OF ORGANIC CARBON ADDITION ON
                      MICROBIAL FLOC FORMATION AND WATER
                      QUALITY IN CLOSED AQUACULTURE SYSTEM

By   Miss. Worarat Vanitchanai

Field of Study   Chemical Engineering

Thesis Advisor   Kasidit Nootong, 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 Master's Degree
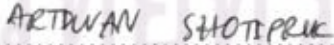
.... Dean of the Faculty of Engineering
(Associate Professor Boonsom Lerdhirunwong, Dr. Ing.)

THESIS COMMITTEE

.... Chairman
(Professor Piyasan Praserthdam, Ph.D.)

.... Thesis Advisor
(Kasidit Nootong, Ph.D.)

.... Thesis Co-Advisor
(Sorawit Powtongsook, Ph.D.)

.... Examiner
(Associate Professor Artiwan Shotipruk, Ph.D.)

.... External Examiner
(Monthon Ganmanee, Ph.D.)

วรรัตน์ วณิชชานัย : ผลจากการเติมสารอินทรีย์คาร์บอนต่อการเกิดตะกอนจุลินทรีย์และคุณภาพ
น้ำในระบบการเลี้ยงสัตว์น้ำแบบปิด (EFFECTS OF ORGANIC CARBON ADDITION ON
MICROBIAL FLOC FORMATION AND WATER QUALITY IN CLOSED
AQUACULTURE SYSTEM) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร. กษิดิศ หนูทอง,
อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. สรวิศ เผ่าทองศุข, 146 หน้า.

งานวิจัยศึกษาการเลี้ยงสัตว์น้ำในระบบปิดโดยนำแนวคิดของระบบเทคโนโลยีไบโอฟล็อกมาใช้
งาน การวิจัยนี้สามารถแบ่งออกเป็น 2 ส่วนคือ (1) การหาสภาวะเหมาะสมของการเกิดไบโอฟล็อกโดย
ไม่มีสัตว์น้ำโดยปรับระดับสัดส่วนของคาร์บอนและไนโตรเจนที่เติมลงในน้ำ และ (2) การเพาะเลี้ยงปลา
นิลในระบบเทคโนโลยีไบโอฟล็อกโดยนำข้อมูลจากในส่วนแรกมาประยุกต์ ผลการทดลองจากส่วน
แรกพบว่าแป้งมันสามารถทดแทนกลูโคสในการสร้างไบโอฟล็อก และการเพิ่มแร่ธาตุที่จำเป็นจาก
อาหารกุ้งยังช่วยกระตุ้นการสร้างไบโอฟล็อกให้ดีขึ้น การเติมอาหารที่สัดส่วน C:N = 16:1 สามารถ
ควบคุมปริมาณแอมโมเนียและไนไตรท์ได้ดีกว่าระดับ C:N ที่ต่ำกว่า นอกจากนี้การใช้น้ำบ่อที่มีไบโอ
ฟล็อกตามธรรมชาติจะสามารถควบคุมคุณภาพน้ำได้ดีกว่าการใช้ประปา สำหรับผลการทดลองในส่วน
ที่สองที่มีการเพาะเลี้ยงปลานิลควบคู่กันพบว่า การเติมแป้งมันและอาหารปลานิลทุกวันลงในถัง
เพาะเลี้ยงในอัตราส่วน C:N = 16:1 จะสามารถควบคุมปริมาณแอมโมเนียและไนไตรท์ได้ดีกว่าชุด
ควบคุมที่เติมอาหารปลาอย่างเดียว ถึงแม้ปริมาณของแข็งแขวนลอยจะเพิ่มขึ้นจาก 30 ถึง 1,118 mg SS/L
การบำบัดไนโตรเจนที่มีประสิทธิภาพจะเกิดขึ้นเมื่อกระบวนไนตริฟิเคชั่นที่สมบูรณ์เกิดขึ้นในถัง ซึ่งมี
นัยว่าน้ำที่นำมาใช้ในระบบเทคโนโลยีไบโอฟล็อกควรถูกปรับสภาพให้พร้อมต่อกระบวนการไนตริ
ฟิเคชั่นที่สมบูรณ์ก่อนนำมาใช้งานจริง ลักษณะของไบโอฟล็อกที่พบในชุดควบคุมและชุดทดลองมี
ความคล้ายคลึงกัน กล่าวคือมีรูปร่างที่ไม่แน่นอนและประกอบด้วยสิ่งมีชีวิตหลายชนิด เช่น แบคทีเรีย
ชนิดเส้นใย โรติเฟอร์ หนอนตัวกลม และจุลสาหร่ายในปริมาณเล็กน้อย การวิเคราะห์องค์ประกอบอย่าง
หยาบของไบโอฟล็อกจากชุดทดลองพบว่ามีปริมาณของ C และ N ที่ 34.5% และ 4.2% ของน้ำหนักแห้ง
ในขณะที่ในชุดควบคุมมีปริมาณของ C และ N ที่ 21.7% และ 2.19% ของน้ำหนักแห้ง การวิเคราะห์
ความหลากหลายของแบคทีเรียในไบโอฟล็อกโดยใช้เทคนิค PCR-DGGE พบว่าในระหว่างชุดควบคุม
และชุดทดลองประชากรแบคทีเรียเด่นของไบโอฟล็อกมีความแตกต่างกัน ข้อมูลดังกล่าวยังชี้ว่าไบโอ
ฟล็อกมีการเปลี่ยนแปลงความหลากหลายของประชากรแบคทีเรียอยู่ตลอดเวลา

ภาควิชา............วิศวกรรมเคมี............ลายมือชื่อนิสิต........วรรัตน์ วณิชชานัย........
สาขาวิชา............วิศวกรรมเคมี............ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..............
ปีการศึกษา............2552............ลายชื่อชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม..............
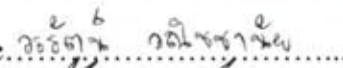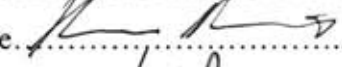
5070431721: MAJOR CHEMICAL ENGINEERING

KEY WORDS: NITROGEN / BIOFLOCS / NITRIFICATION/ TILAPIA

WORARAT VANITCHANAI: EFFECTS OF ORGANIC CARBON ADDITION ON MICROBIAL FLOC FORMATION AND WATER QUALITY IN CLOSED AQUACULTURE SYSTEM. THESIS ADVISOR: KASIDIT NOOTONG, Ph.D., THESIS CO-ADVISOR: SORAWIT POWTONGSOOK, Ph.D., 146 pp.

This research examined the closed-water aquacultures by using the concept of biofloc technology. The first part of research studied the optimal condition for biofloc formation based on manipulating the substrate C:N ratio while the latter part used the information obtained from the first part to cultivate tilapia in suspension systems without any water exchange. The research also investigated the characteristics of biofloc. For the first section, tapioca starch can be substituted for glucose as means to stimulate biofloc formation. With the presence of essential elements in shrimp diets the biofloc production was more productive. The weight C:N ratio at 16:1 was able to promote the highest biofloc production and was efficient in maintaining TAN and nitrite compared to the lower C:N ratios. Pond water containing natural biofloc appeared more effective than tap water in controlling inorganic nitrogen. For the second part of this research, the daily use of C:N ratio at 16:1 was more effective in maintaining TAN and nitrite in water. Despite a significant increase of suspended solids from 30 to 1,118 mg SS/L, the effective nitrogen treatment did not proceed until a complete nitrification was established in the tanks, thereby implying that the water must be pre-acclimated to achieve the complete nitrification process. The morphology of biofloc was irregular shape containing filamentous microorganisms, rotifers, nematode, and small amount of microalgae. The proximate analysis revealed that the carbon and nitrogen contents of biofloc in the treatments were at 34.5% and 4.2%, respectively whereas the carbon and nitrogen contents in the controls were at 21.7% and 2.19%. Finally, the nitrogen balance and PCR-DGGE analysis indicated that biofloc were highly diverse and dynamics.

Department: ...Chemical Engineering... Student's Signature..........................

Field of Study: Chemical Engineering... Advisor's Signature..........................

Academic Year: ....2009.............. Co-Advisor's Signature..........................

ACKNOWLEDGEMENTS

This thesis will never have been completed without the help and supports of many people who are gratefully acknowledged here. Firstly, I would like to express my sincere gratitude to Dr. Kasidit Nootong and Dr. Sorawit Powtongsook, my advisor and co-advisor, for their suggestions, guidance, warm encouragement and generous supervision throughout my master program. I am also grateful to Professor Piyasan Praserthdam, Associate Professor Artiwan Shotipruk and Dr. Monthon Ganmanee for their valuable suggestion and specific guidance to improve my thesis.

For my colleagues, I would like to thank Miss. Maliwan Kutako, Mr. Seri Donneua and other friends at the Department of Chemical Engineering of Chulalongkorn University and at the Center of Excellence for Marine Biotechnology of Chulalongkorn University for their sincere friendship and assistance during my research work. Finally, I would like to acknowledge the Thailand Research Fund, the national Innovation agency and Manit Farm for their financial supporting. Without their funding, my thesis is impossible.

Finally, I would like to express me sincere my sincere indebtedness to my family for their worth supports throughout my Master courses.

CONTENTS

LIST OF TABLE

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Motivations

Aquaculture is the cultivation of aquatic animals and plants as means to produce food for human consumption. Aquaculture can be done in various scales, ranging from small earthen ponds for food production in rural area to large commercial scale to meet export demand. Aquaculture is a rapidly growing food producing sector that has grown at an average of 8.9% per year since 1970 compared to only 1.2% for captured fisheries and 2.8% for terrestrial farmed meat-production systems over the same period (FAO, 2004). Despite the rapid growth, aquacultures still need to increase at least 5-folds to satisfy the requirement for human food consumption particularly in the developing countries (Subasinghe, 2005; Gutierrez-Wing and Malone, 2006; Matos et al., 2006).

Aquacultures in Thailand are carried out generally in earthen ponds or in cages. These practices require significant amount of water from natural resources, which are often affected by diseases or waste discharges generated upstream from both domestic and industrial sources. Accumulation of wastes produced during aquaculture cultivation can also cause negative health effects on farmed animals, and the release of production water without proper treatment is able to create adverse environmental concerns namely ammonium toxicity to fish, eutrophication and oxygen depletion in receiving water (Timmons, et al., 2002; Tchobanoglous et al., 2004). For these reasons, the aquaculture industry in Thailand begins to shift its production strategy from extensive open-ponds towards intensive closed or semi-closed systems, which treat and recycle water within farms. In Thailand, the closed or semi-close aquaculture systems are normally found in biosecured facilities, which strictly control the disease transmitting, or in those shrimp producing farms, which received GAP (Good Aquaculture Practice) or CoC (Code of Conduct for Aquaculture) from the Department of Fisheries.

Aquaculture ponds can be categorized into 3 types; outdoor earthen ponds, outdoor lining ponds, and indoor pond. Outdoor earthen ponds are popular among Thai farmers, while outdoor lining ponds require the synthetic materials such as HDPE sheets or cements to cover their soil sediments. Indoor ponds are similar to outdoor lining ponds but are largely limited by the availability of light. By excluding the natural factors such as light, rain and temperature, it was apparent that the water quality within aquaculture ponds is directly related to production aspect. Excessive accumulation of inorganic nitrogenous compounds especially ammonium and nitrite is undesirable and yet often encountered in both outdoor lining ponds and indoor ponds. These inorganic nitrogenous compounds are generated from animal excretion and biological degradation of unconsumed feeds (Avnimelech and Ritvo, 2003). Buildup of ammonium and nitrite above 1.0 mg N/L is generally known to cause adverse health effects towards aquatic stocks including a higher stress, a lowering oxygen transport capability in blood, a weakening immune system or even death (Crab et al., 2007).

Many treatment systems to control inorganic nitrogen toxicity are available. Phytoplankton-based treatment systems are attractive due to their simplicity and low operational cost but often fail to sustain a stable operation because of the periodic phytoplankton bloom and crash (Hargreaves, 2006). Nitrifying biofilters are more reliable and have been successfully tested for various aquacultural applications. Despite many advantages, the use of nitrifying biofilters is susceptible under an outdoor operation and more importantly the systems are unable to reutilize expensive unconsumed proteins in feeds. The ability to recycle proteins is an important aspect for the sustainability to aquacultures as the aquatic animals are able to incorporate the available proteins in feeds on average only 25 to 30% (Avnimelech and Ritvo, 2003). Biofloc technology systems currently receive more attention particularly for the closed-water shrimp and Tilapia cultivation since they feature the wastewater treatment and the feed protein recycle simultaneously. In biofloc technology systems, the removal of ammonium is performed based on an enhancement of heterotrophic bacterial growth to assimilate nitrogen and incorporate it into new cellular proteins (Avnimelech, 2006). As bacteria in the water flourish reaching a high density, they tend to form noticeable aggregates (i.e., biofloc), which in turn can be consumed by aquacultures as natural food source (Burford and Lorenzen, 2004). Addition of

carbon and nitrogen sources at high C:N ratio into aquaculture systems has been recommended as the controlling element to establish the biofloc (Avnimelech, 2006). After an extensive literature search, works in biofloc technology in Thailand is extremely limited and hence it is appropriate to initiate the research that develops the biofloc technology system that is inexpensive and suitable for the tropical climate found in Thailand. Specifically, this work intends to determine the optimal conditions for biofloc formation and then using the obtained information to cultivate aquacultures without any water exchange. Additional information about the ability of the system to control the concentrations of ammonium and nitrite, the compound dynamics during the system startup and the biofloc characteristics are also assessed.

1.2 Objectives

1. Determine the optimal condition in term of the quantity of carbon and nitrogen sources required to achieve the maximum biofloc formation.

2. Apply the optimal condition for the biofloc formation to the zero-water exchanged aquaculture and assess the ability of the biofloc technology system in maintaining acceptable ammonium and nitrite concentrations.

3. Identify the biological processes that are responsible for the nitrogen control in the biofloc technology systems, and study the physical and chemical properties of biofloc.

1.3 Scopes of work

1. The experiment is carried out at the Center of Excellence for Marine Biotechnology at the Chulalongkorn University and at the Department of Chemical Engineering of Chulalongkorn University.

2. The experiment is located outdoor next to the laboratory building. The experimental system is covered with transparent plastic sheet to partially allow sunlight and avoid rainwater penetration.

3. For the determination of the optimal biofloc formation that is performed without fish culture, the substrate C:N ratios ranging from 2:1 to 16:1 are chosen to investigate the biofloc formation. The best condition obtained earlier is employed for the zero-water exchanged Tilapia cultivation in the suspension systems. The experiment used male Tilapia with the initial weight from 25 to 40 g and stocked at the initial biomass about of 3.0 kg/m^3. Investigate the biofloc characteristics in term sizes and proximate composition (C, H and N). Perform the nitrogen balance at the end of the Tilapia cultivation to determine the extents of various processes in controlling ammonium and nitrite toxicities.

4. The following variables are constantly monitored: the concentration of NH_4^+-N, NO_2^--N and NO_3^--N, biofloc volume, chlorophyll, total solid (TS) and suspended solid (SS). The biofloc characteristics were examined by using the conventional microscope and the fluorescent microscopy.

1.4 Benefits

1. The obtained information can be used as guidelines to establish the biofloc technology systems under the Thai climate.

2. The result may be applied as the strategy for sustainable aquacultures.

3. The knowledge obtained can be transferred to local Thai aquaculturists.

CHAPTER 2

LITERATURE REVIEW

2.1 Intensive aquacultures

Achieving higher production yield is a common goal for today aquacultures. However, intensification requires costly investment and operational expense. Evolution of pond intensification can be summarized in Table 2.1. Aquacultures (fish and shrimp) can be grown at very high density in aerated-mixed ponds. However, with increasing aquaculture biomass, water quality becomes a limiting factor due to accumulation of toxic compounds particularly ammonia and nitrite. Therefore, in order to use aerated-mixed ponds to grow aquacultures, water quality must be controlled. Three different approaches were used to control water quality: (1) exchanging pond water with freshwater at high rates at greater than 5 times per day (2) treating and recycling water through external biofilters and (3) treating water within pond using algae or activated bacterial communities.

Table 2.1 Schematic presentation of pond intensity levels, approximate annual fish yields and limiting factors (Avnimelech, 2006)

Pond type	Intervention	Yields (kg/ha/year)	Limiting factors
Minimal Feed	Minimal feed with grains, farm and home residues	< 2000	Limits of primary production food chain efficiency
Fed ponds	Feeding by complete diet pellets	2,000 – 4,000	Early morning oxygen depletion
Night time aeration	Night time or emergency aerators, ~1-5 hp/ha	4,000 – 10,000	Sludge accumulation, anaerobic pond bottom
Intensive mixed aerated ponds	24 h aeration, >20 hp/ha, well-mixed	20,000-100,000	Water quality control

2.2 Important water quality parameters

In intensive aquacultures, a basic knowledge about water chemistry is critical for the success of any intensive operation. Table 2.2 lists important water quality parameters required for aquacultures.

Table 2.2 Criteria for water quality parameters in aquaculture (Modified from Timmons et al., 2002)

Parameters	Concentration (mg/L)
Alkalinity (as $CaCO_3$)	50 – 300
Ammonia (TAN) Cool-water fish	< 1.0
Ammonia (TAN) Warm-water fish	< 3.0
Carbon Dioxide (CO_2)	20 – 60 depending on species
Chlorine (Cl)	< 0.003
Hydrogen sulfide (H_2S)	< 0.002
Nitrite (NO_2)	< 1.0
Nitrate (NO_3)	0 – 400
Oxygen Dissolved (DO)	> 5
Ozone (O_3)	< 0.005
pH	6.5 – 8.5
Phosphorous (P)	0.01 – 3.0
Salinity	depends on salt or fresh species
Sodium (Na)	< 75
Sulfate (SO_4)	< 50
Sulfur (S)	< 1.0
Total suspended solids (TSS)	< 80

2.3 Tilapia

2.3.1 Characteristic of Tilapia

Tilapia *(Orechromis niloticus)* is a native animal of African continent. Tilapia has fairly conventional, laterally compressed and deep body shape. The body is covered with relatively large, cycloid scales, which are not easily dislodged (Ross, 2000). The dosal and anal fins have hard spines and soft anterior in an advanced configuration. The numbers of scales, vertebrae, gill rakers and fin raya and spinners are widely used for species distinction and identification. Tilapia bodies are generally characterized by vertical bars, with relatively subdued colors and with little contrast over the body colors. This provides the fish with a modest ability to change their colors, in response to stress by controlling skin chromatophores.

2.3.2 Tilapia cultivation

Tilapia cultivation can be categorized into 3 groups based on the initial density of the crops, the quantity of feed, and operation (Abdel-Fattahm and El-Sayed, 2006).

1. Extensive Culture. Extensive culture aims for domestic consumption. Tilapia acquires natural foods available within pond (i.e., earthen ponds) so that it is unnecessary to provide additional diets. The initial Tilapia density ranges from 0.5 to 2 Tilapia/m^2.

2. Semi-intensive Culture. Semi-intensive culture is done purposely for domestic consumption as well as commercialization. Tilapia acquires natural foods available within ponds and may need supplemental diets occasionally. The initial Tilapia density for semi-intensive culture is estimated from 2 to 4 Tilapia/m^2.

3. Intensive Culture. Intensive culture requires high quality feeds, pond maintenance, heavy aeration and disease control. Intensive culture also needs significant water replacement several times per day to maintain good water quality. For these reasons, intensive culture is carried out purposely for commercialization. The initial Tilapia density for intensive culture is from 4 to 10 Tilapia/m^2.

4. Caged Production. Caged production is often found in Thailand at the moment. Cages, made from synthetic materials, are normally available in square, rectangular or spherical shapes. Different shapes of cages influences the characteristic of water flow, quantity of incoming water and solid-deposition. In Thailand, square shaped (1.2 x 1.2 x 2.5 m) and rectangular shaped (4 x 2 x 2.5 m) are popular. Deployment of Tilapia weighed from 50 to 60 g was conventional to reach an initial crop density around 4 to 6 kg/m^3.

2.3.3 Conditions affecting Tilapia growth

Tilapia grows well in the temperature range from 20 to 35 ℃ (Balarin and Haller, 1979). Tilapia stops eating when the temperature is lower than 15 °C and dies at the temperature below 8 °C (Abdel-Fattahm and El-Sayed, 2006). Tilapia should be cultivated in water with alkalinity from 200 to 300 mg/L CaCO$_3$ (Abdel-Fattahm and El-Sayed, 2006). The suitable pH for Tilapia was reported from 6.5 to 8.5 (Ross, 2000). Inorganic nitrogen compounds. Ammonium is toxic towards Tilapia when its concentration exceeded 1.0 mg N/L. The threshold for nitrite was reported at 2.1 mg N/L but it was recommended to keep nitrite concentration below 1.0 mg N/L (Balarin and Haller, 1979).

2.4 Nitrogen in aquaculture pond
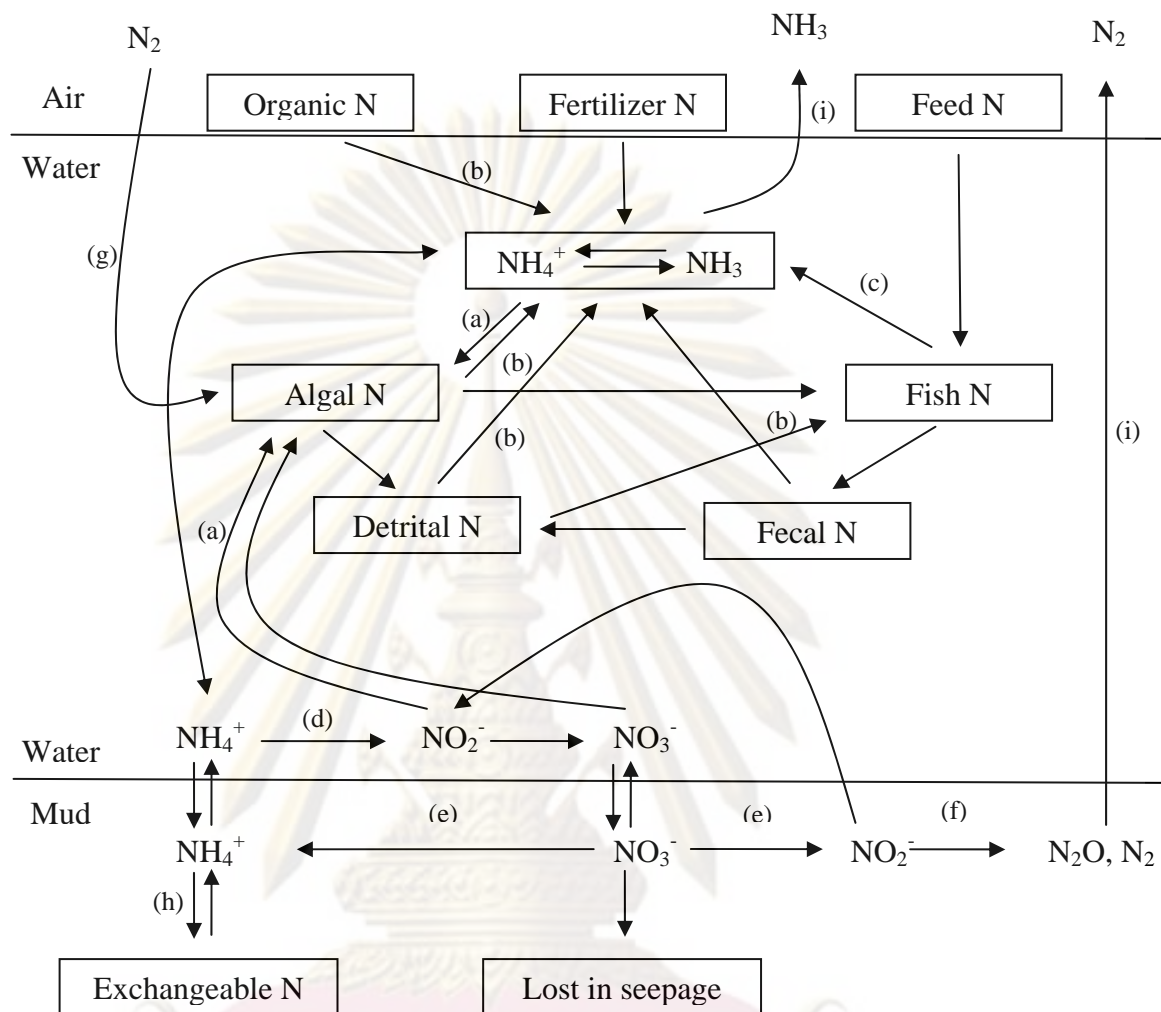
Figure 2.1 The nitrogen cycle in aquaculture ponds. The illustration is simplified by omitting the food chain between algae and fish. Major processes illustrated are (a) assimilation (b) mineralization (c) excretion (d) nitrification (e) nitrate reduction (f) denitrification (g) biological nitrogen fixation (h) absorption of ammonia in mud cation-exchange reactions and (i) ammonia volatilization. (Modified from Boyd and Tucker, 1998)

Nitrogen is a major nutrient affecting the productivity of aquatic ecosystems since it is an essential component of protein and other constituents of cellular

protoplasm. Aquatic animals meet their nitrogen requirement by obtaining food produced naturally within ponds or by feeding from aquaculturists. Figure 2.1 illustrates the nitrogen cycles in aquaculture ponds. Clearly, the nitrogen input are from various sources such as feeding, biomass decay or aquatic animal excretion; and undergoes many biological reactions both in water column and in sediments to change their forms. These biological reactions are essential for the natural water treatment in the ponds and are used as the basis for the design of water treatment and circulating systems for commercial aquacultures. Important aspects of Figure 2.1 that is related to the development of aquaculture systems can be summarized as followed:

2.4.1 Inorganic nitrogen compounds and toxicities

2.4.1.1 Ammonia

Ammonia is introduced into aquaculture ponds via feeds, aquatic animal excretion, and biological degradation of unconsumed feeds. Ammonia is available in water in two forms (NH_3 or NH_4^+) depending on pH of water. Free ammonia (NH_3) is more toxic towards aquatic animal in comparison to ionized form (NH_4^+). The proportion of free ammonia increases with increasing pH and increasing temperature. Toxic concentrations of ammonia can damage gills of fish, consequently impairing its respiratory system. Ammonia also causes the neurological and cytological failure in fish (Nootong, 2006). The acceptable level of ionized ammonia (NH_4^+) is 1.0 mg N/L.

2.4.1.2 Nitrite

The presence of nitrite in water is generally trivial as it is the intermediate of nitrification process, which converts ammonium into nitrate. However, nitrite accumulation in water is possible due to incomplete nitrification and denitrification, and its consequence is undesirable. Nitrite can combine with Fe^{2+} in hemoglobin forming a compound called methamoglobin, which possesses a lower oxygen transport capability than hemoglobin. The presence of nitrite at high concentration

can cause a lack of oxygen in Tilapia. In human, nitrite is a potential carginogic compounds. Infant under the age of 6 months may become seriously ill and die, if untreated, after drinking water containing nitrite (Nootong, 2006). For the purpose of aquaculture, it is desirable to keep nitrite concentration under 1.0 mg N/L.

2.4.1.3 Nitrate

Nitrate is a stable compound, which is an end-product of nitrification. Nitrate, although far less toxic than ammonium and nitrite, can become toxic towards Tilapia when its concentration exceeds 70 mg N/L (Van Rijn, 1996). Nitrate is poisonous to human especially in baby under 4 to 6 months old because it can bind with hemoglobin to form methamoglobin. Discharge of nitrate into natural water resources can cause eutrophication, which is a natural aging of freshwater reservoir such as lakes to become organically rich, thereby leading to domination of weeds and eventually transforming into mash land (Tchobanoglous, et al., 2004). Discharge of nitrate into natural water resource quickly accelerates eutrophication by stimulating the growth of microalgae.

2.4.2 Biological processes for inorganic nitrogen treatment

Treatment of inorganic nitrogen compounds (i.e., ammonia, nitrite and nitrate) can be accomplished by different biological processes in the nitrogen cycles. Common biological processes for inorganic nitrogen treatment include nitrogen assimilation, ammonification, nitrification, heterotrophic denitrification and recently discovered anaerobic ammonium oxidation (Anammox).

2.4.2.1 Nitrogen assimilation

Nitrogen assimilation can be defined as the process in which nitrogen is acquired into cells to form new cell constituents (i.e., biomass). Nitrogen assimilation by phytoplankton is important for inorganic nitrogen treatment. Assimilated nitrogen is incorporated into proteins of new biomass during photosynthesis. Hargreves (1998)

estimated that microalgae with the composition C:N:P of 106:16:1 was capable of assimilating inorganic nitrogen into cells from 150 to 450 mg N/m^2/day at low temperature and from 750 to 1,500 mg N/m^2/day at high temperature. Microalgae was able to utilize all forms of nitrogen in water but appeared to prefer ammonium and nitrite (DeBoer, 1981). Hargreves (1998) further pointed out that microalgae would first assimilate ammonium until its concentration is less than 0.3 mg N/L before switching to acquire nitrate. Heterotrophic bacteria can also incorporate ammonium and nitrite to synthesis new proteins during cell growth. Addition of organic carbon compounds can quickly enhance assimilating process given that oxygen is available in sufficient quantity. Nitrogen assimilation by heterotrophic bacteria and microalgae can be described by equation 2.1 and 2.2, with the symbols $C_5H_7O_2N$ and $C_{106}H_{263}O_{110}N_{16}P$ representing chemical compositions of heterotrophic bacteria and microalgae, respectively (Ebeling and Timmons, 2007).

$$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2$$

$$\rightarrow C_5H_7O_2N + 6.06H_2O + 3.07CO_2 \quad \text{(Bacteria)} \quad (2.1)$$

$$16NH_4^+ + 92CO_2 + HPO_4^{2-} + 92H_2O + 14HCO_3^-$$

$$\rightarrow C_{106}H_{263}O_{110}N_{16}P + 106O_2 \quad \text{(Microalgae)} \quad (2.2)$$
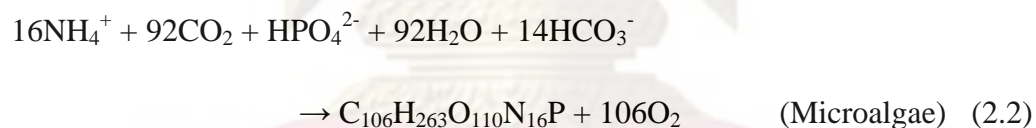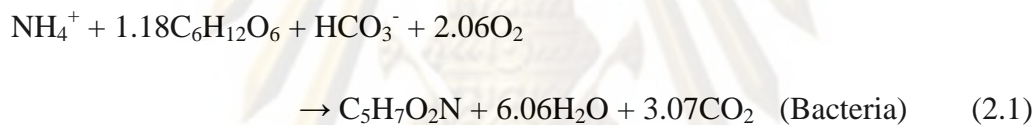
2.4.2.2 Ammonification

Ammonification is the release of ammonia from organic matters (e.g., proteins and urea). Ammonification is an important mechanism that is responsible for feed degradation in aquaculture ponds. Proteins in feeds are broken down by bacteria into constituent amino acids and the subsequent degradation gives ammonia.

2.4.2.3 Nitrification

Nitrification is the biological process that converts ammonia successively into nitrite and nitrate. Microorganisms responsible for nitrification are chemoautotrophic

nitrifying bacteria, which are known to utilize inorganic carbon and ammonia as carbon and energy sources, respectively. The first step of nitrification, which involves the conversion of ammonium to nitrite, is carried out by ammonium oxidizing bacteria (AOB) such as *Nitrosomonas, Nitrosolobus, Nitrospira, Nitrosococus,* and *Nitrovibrio* (Nootong, 2008). Nitrobacter is commonly recognized as bacterial species responsible for the second step of nitrification that is the conversion of nitrite to nitrate. Other nitrite oxidizing bacteria (NOB) include *Nitrospina* and *Nitrococcus*, but they are marine-obligated. Recently, *Nitrospira*-like bacteria was found as common NOB in various wastewater treatment facilities (Nootong, 2006). Equation 2.3 and 2.4 represent nitrifying reactions by AOB and NOB.

$$NH^+_4 + 1.5\ O_2\ \rightarrow\ NO_2^-\ + 2H^+ + H_2O \qquad \text{(AOB)} \qquad\qquad (2.3)$$

$$NO^-_2 + 0.5O_2 \rightarrow NO_3^- \qquad \text{(NOB)} \qquad\qquad (2.4)$$
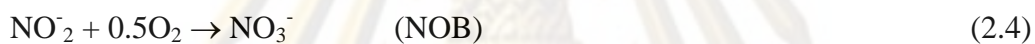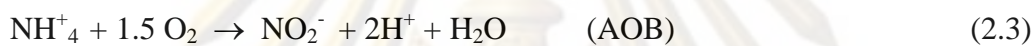
The following characteristics of nitrification need to be mentioned (1) the C:N ratio of nitrifying bacterial biomass is 4.29 g C/g N; the net production of biomass is very small at 0.20 g VSS/g N; the biomass (i.e., VSS) contents consist of 53.1% carbon and 12.4 % nitrogen; nitrifying reaction utilizes alkalinity as the primary carbon source at 7.05 g $CaCO_3$ per gram of nitrogen; and the actual carbon requirement, oxygen requirement and carbon dioxide produced are 1.69 g C/g N, 4.18 g O_2/g N and 5.85 g C/g N, respectively.

Many environmental factors can affect nitrification. Effective nitrification was observable when the dissolved oxygen (DO) concentration is greater than 2.0 mg/L (Nootong, 2008). Pure cultures of *Nitrosomonas* and *Nitrobacter* exhibited a stoppage of nitrification when DO is lower than 0.5 mg/L. Temperature can also influence the rate of nitrification. Freshwater nitrifying bacteria were reported to grow at the temperature range from 8 to 36 ºC, with the optimal temperature at 30 ºC (Bitton, 1994). Marine nitrifying bacteria were reported to have optimal temperature range from 30 to 35 ºC (Bitton, 1994). Temperature dependency of nitrification can be described Arrhenius equation. The optimal pH for nitrification is reported in the

range between 7.0 and 8.5. The pH value lower than 6.0 was reported to display an inhibitory effects on nitrification (Nootong, 2006). The extent of organic carbon in water can also affect the success of nitrification. Increasing the BOD:N ratio (i.e., increasing organic content) can stimulate the growth of heterotrophic bacteria, which possess higher oxygen affinity (Sharma and Ahler, 1977). The rate of nitrification was reported to decrease as high as 20 to 29% when the organic matters, measured as the chemical oxygen demand (COD) increased from 2 to 6 kg/m^3/day (Tchobanoglous, et al., 2004). Finally, compounds such as organic matters, heavy metals, cyanide, thiourea, cresol, phenol, anilines, mercaptan, pesticide and halogenated compounds were able to partially and even completely inhibit nitrifying bacteria (Lu et al., 1984; Sato et al., 1988; Bitton, 1994).

Table 2.3 Examples of inhibitory compounds for nitrification (Adapted from Lehr and Keeley, 2005).

Chemicals	Inhibitory Concentrations (mg/L)
Cobalt	0.08 – 0.5
Chromium	0.25
Copper	0.05 – 0.56
Nickel	0.25
Zinc	0.08 – 0.5
Cadmium	14.3
Sulfide	5.0
Sodium Chloride	35,000
Sodium Cyanide	100
Hydrogen Sulfide	50
Sodium Cyanide	1
Potassium Dichromate	6.0

2.4.2.4 Heterotrophic denitrification

Heterotrophic denitrification is a biological process in which nitrate is reduced into nitrogen gas by denitrifying bacteria under oxygen-limited or anaerobic conditions. Oxygen is the preferred electron acceptor for bacteria under aerobic condition. In contrast, nitrate is the second preferred choice, and in lights of nitrification where nitrate is abundant and oxygen is limited, denitrifying bacteria is expected to utilize nitrate as the electron acceptor. Common denitrifying bacteria are diverse including *Achromobacter, Bacillus denitricans*, *Flavobacterium, Micrococcus denitrificans, Dinitrobacillus, Spirillum* and *Pseudomonas stutzeri* (US EPA., 1975; Anderson and Ibahim, 1978; Knowler, 1982). Denitrifying bacteria also require electron donors, which are normally organic carbon compounds such as methanol, ethanol and acetate, to provide carbon as energy source. Among available choices, methanol is the most popular due to its price. If using methanol as the electron donor, denitrifying reaction can be written as shown in equation 2.5. According to equation 2.5, denitrification will increase the pH of water since it generates hydroxyl ion.

$$6NO_3^- + 5CH_3OH \quad \rightarrow \quad 5CO_2 + 3N_2 + 7H_2O + 6OH^- \qquad (2.5)$$
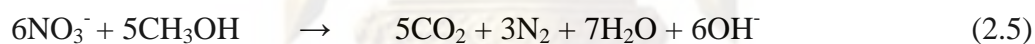
Many environmental factors can affect denitrification including DO, temperature, pH and inhibitory compounds. Low DO must be kept in order to sustain successful heterotrophic denitrification. Many reports suggested different threshold for the DO concentration ranging from 0.2 to 2.0 mg/L but generally accepted DO values should not exceed 0.5 mg/L (Christensen and Harremoes, 1977). Similar to nitrification, temperature dependency of heterotrophic denitrification can be described by Arrhenius equation. Denitrification is active under wide temperature range from 0 to 50 ºC with the optimal values reported between 35 and 40 ºC (Winker, 1984; Bitton, 1994). It is agreeable that the optimal pH range for heterotrophic denitrification is between 7 and 8 (Winkler, 1984). The rate of denitrification decreases approximately 30% when the pH is outside that range. The pH levels can also determine the end products of the process: the majority of end product is nitrous oxide when the pH is under 7.3 while nitrogen gas is dominant beyond that pH level.

Heterotrophic denitrification is inhibited by many substances such as acetylene, pesticides and nitrifying inhibitors. Sulfide inhibits nitric oxide and nitrous oxide reduction process. Metal chelating agents such as potassium cyanide, dithiol and o-phenanthroline inhibits nitrate reductase in denitrifying bacteria.

2.4.2.5 Anaerobic ammonium oxidation (Anammox)

Anaerobic ammonium oxidation (Anammox) is a biologically process that autotrophically converts ammonium to nitrogen gas with nitrite as a terminal electron acceptor. Reduction process to produce nitrogen gas is generated without organic carbon requirement (Khin and Annachhatre, 2004). Anammox has the disadvantage since the bacteria responsible for this process, *Plantomecetales*, have an extremely slow growth with the doubling time about 11 days (Khin and Annachhatre, 2004). However, the quality of wastewater that was used during Anammox research contained extremely high concentrations of ammonium (i.e., low C:N ratio), a characteristic that is in contrast to aquaculture wastewater (Nootong, 2008). The optimal conditions for anammox are similar to those for nitrification.

2.5 Inorganic nitrogen treatment for aquaculture systems

Biological processes described in section 2.4.2 are the basis for the design of treatment systems for the closed-water aquaculture application. Based on literature reviews, inorganic nitrogen treatment systems for aquaculture applications can be classified as the attached-growth and suspended-growth systems.

2.5.1 Attached-growth systems

In attached-growth system, bacteria are immobilized via adsorption onto the surface of cell supporting materials called biofilters. Examples of biofilters include stone, marble, sand or plastic such as PVC, polyethylene and polypropylene. Plastic biofilters are increasingly popular due to their durability and high surface area to encounter nitrifying bacterial slow growth. Plastic biofilters are available

commercially for example Biocord[TM], Bioball, HyperDrain[TM] and etc. Attached-growth systems can be further divided into nitrifying and denitrifying systems.

2.5.1.1 Nitrifying systems

Nitrogenous wastewater from aquaculture cultivation is normally circulated through aerated nitrifying biofilters located outside production ponds. Dissolved oxygen concentration above 4.0 mg/L and suspended solid removal are always maintained to ensure optimal growth for nitrifying bacteria and aquatic stocks. The disadvantage of nitrifying systems usually involves an expensive operational expense and clogging of suspended solids between biofilters pored spaces. Many design configurations of nitrifying biofilters are available including rotating biological contactor (RBC), trickling filters, fluidized filters and microbead filters.

In rotating biological contactors (RBC), immobilized cells in the form of biofilm are attached on stationary surfaces of large plastic discs amounted on a horizontal shaft rotating slowly from 2 to 5 rpm (Tchobanoglous, et al., 2004). Because of the rotation, different sectors of the discs are alternatively exposed to oxygen and wastewater, thus allowing nitrification to proceed. RBCs were able to reduce the clogging of suspended solids (Brazil, 2006). The rates of inorganic nitrogen removal by RBCs were reported in the range from 0.19 to 0.79 g TAN/m^2/day (Brazil, 2006; Crab et al., 2007).

Tricking filters consists of a bed of highly permeable medium to which microorganisms are attached and through which wastewater is percolated. Filter media usually consist of stones or light plastic packing materials, which possess the specific surface area from 100 to 1,000 m^2/m^3 (Crab et al., 2007). Wastewater from aquaculture ponds is introduced evenly at the top of media bed and trickles down between media pore space so that oxygen and inorganic nitrogen mass transfers can take place. The disadvantage associated with trickling filters is clogging from suspended solids in wastewater and from overgrowth of biofilms. The rates of inorganic nitrogen removal by trickling filters were reported in the range from 0.24 to 0.64 g TAN/m^2/day (Eding et al., 2006; Crab et al., 2007).

The principle of fluidized filters is similar to that of the trickling filters. Nitrification is carried out on the surface of cell supporting materials (e.g., sand and polystyrene beads) with average sizing from 1 to 3 mm. Fluidized sand filters have the specific surface area for immobilization in the range from 4,000 to 20,000 m^2/m^3 (Shieh and Keenan, 1987). The wastewater is introduced upward at the bottom of bioreactor column at high rate to fluidize cell supporting materials. Fluidized filters are able to treat large volume of wastewater and less susceptible to solid clogging. However, fluidized filters are energy intensive and need efficient external aeration system to maintain aerobic condition within expanded bed. The rates of inorganic nitrogen removal by fluidized filters were reported in the range from 0.19 to 0.79 g TAN/m^2/day (Sandu et al., 2002; Summerfelt and Sharrer, 2004; Crab et al., 2007).

Design of microbead filters is similar to tricking filters but the size of beads (i.e., cell supporting material) is smaller. Size of bead varies from 1 to 3 mm, thereby giving the specific surface area from 1,360 to 3,780 m^2/m^3 (Greiner and Timmons, 1998). Wastewater is distributed evenly over the top of the packing column. Inorganic nitrogen treatment occurs in biofilm layers formed on the surface of beads. At the same time, suspended solids are trapped between void spaces. Microbead filters are capable of treating large volume of wastewater and separation of suspended solid. The rates of inorganic nitrogen removal by microbead filters were reported in the range from 0.3 to 0.6 g TAN/m^2/day (Greiner and Timmons, 1998; Sastry et al., 1999; Crab et al., 2007).

Table 2.4 Various types and operating results of nitrifying biofilters for Tilapia cultivation system.

Intial density	Type of biofilters	Rate of biofilters	Reference
2.4 kg/m^3	Floating bead filter Type polyethylene and Rotating biological contactor	56.2 mg TAN/m^2/day (Bead filters) 257 mg TAN/m^2/day (RBC)	DelosReyes and Lawson, 1996
0.39 kg/m^3	Rotating Drum filter	330 mg TAN/m^2/day	Twarowska et al., 1997
20.0 kg/m^3	Submerged biofilter	3.46 mg TAN/m^2/day	Al-hafedh et al., 2003
10.3 kg/m^3	PP plastic chips PE blocks	46.5 mg TAN/m^2/day 44.5 mg TAN/m^2/day	Mohanmmad and Emmanuel, 2000
Not specify	Floating bead filter	54 mg TAN/m^2/day (series filter) 81 mg TAN/m^2/day (Solitary filter)	Hargrave, 1998
Not specify	Bubble-washed bead filter	0.33 g TAN/m^2/day	Sastry et al., 1999
Not specify	Trickling filter	0.2 g TAN/m^2/day	Lekang and Kleppe, 2002
140 kg/m^3	Microbead and Trickling filters 53 m^3 rearing tank	130 mg TAN/m^2/day 940 mg TAN/m^2/day	Greiner and Timmons., 1998

2.5.1.2 Integrated nitrifying and denitrifying systems

Despite being relatively harmless to aquatic species, nitrate at extremely high concentrations may induce stress on aquacultures and create environmental concerns if proper treatment is not met. Denitrification occurs naturally in sediments. However, natural process cannot handle large volume of aquaculture wastewater containing high nitrate concentrations. Literature reviews indicate limited

information about combined nitrifying and denitrifying systems for closed aquacultures. The design features of denitrifying systems are similar to those for nitrification in the way that it requires high surface area biofilters to immobilize denitrifying bacteria. Additional feature of denitrifying systems is the quick oxygen removal from wastewater to ensure anaerobic condition. It is desirable to keep DO concentration in denitrifying bioreactor below 1.0 mg O_2/L. The research and development of combined nitrifying and denitrifying systems in Thailand was reported by Triyarat (2003). In this work, the tubular denitrification bioreactor was developed. Effluent containing nitrate (DO > 4.0 mg O_2/L) from nitrifying bioreactor was slowly introduced into long cylindrical tube filling with plastic Bioball[TM] biofilters. Methanol, chosen as an electron donor, is delivered via automated ORP control at the beginning section of the tube as mean to remove oxygen in wastewater. The performance of the system, tested with the closed-water shrimp cultivation, showed that the tubular bioreactor was able to grow shrimp without any water exchange for 7 months. The average ammonium concentration was observed below 0.06 mg N/L without any significant nitrite accumulation. The maximum nitrate concentration in this works was reported at 39 mg N/L.

2.5.2 Suspended-growth systems

In suspended-growth system, microorganisms (e.g., bacteria and microalgae) are free to move within water. Due to the slow growth of nitrifying and denitrifying bacteria, the use of suspended growth in treating inorganic nitrogen compounds is limited. Based on literature reviews, it becomes clear that nitrification, denitrification and direct nitrogen assimilation can occur simultaneously in suspended-growth systems. Examples of existing suspended-growth systems are earthen stabilization ponds and biofloc technology ponds.

2.5.2.1 Earthen stabilizing ponds

Earthen stabilizing ponds or in short as earthen ponds are the least expensive system to build and maintain. Wastewater from aquaculture ponds is introduced into earthen pond with the dept about 0.5 to 1.0 m and thoroughly mixed to attain

homogeneity. Wastewater is kept in earthen from 1 to 2 days or as long as a week to ensure a complete treatment. Figure 2.2 displays the biological relationships between different biological processes in earthen ponds. Oxygen is generated from reaeration at water surface and from photosynthesis of phytoplankton during the day. Oxygen is consumed during nitrification or aerobic degradation to produce ammonium, CO_2, NO_3^- and PO_4^{3-}. Microalgae utilize these inorganic compounds for growth. Nitrate removal is accomplished via direct assimilation or denitrification at bottom sediments to produce nitrogen gas. These biological processes are cyclic so that inorganic nitrogen waste can be treated continuously. The capability of earthen ponds depends strongly on the rate of oxygen production by microalgae as well as the ability of both nitrifying and denitrifying bacteria to utilize nitrogen. Earthen ponds with microalgae are capable of treating inorganic nitrogen waste under wide ranges from 176 to 2,113 mg N/m^{2}day (Brune et al., 2003; Burford et al., 2003; Hargreaves, 2006).
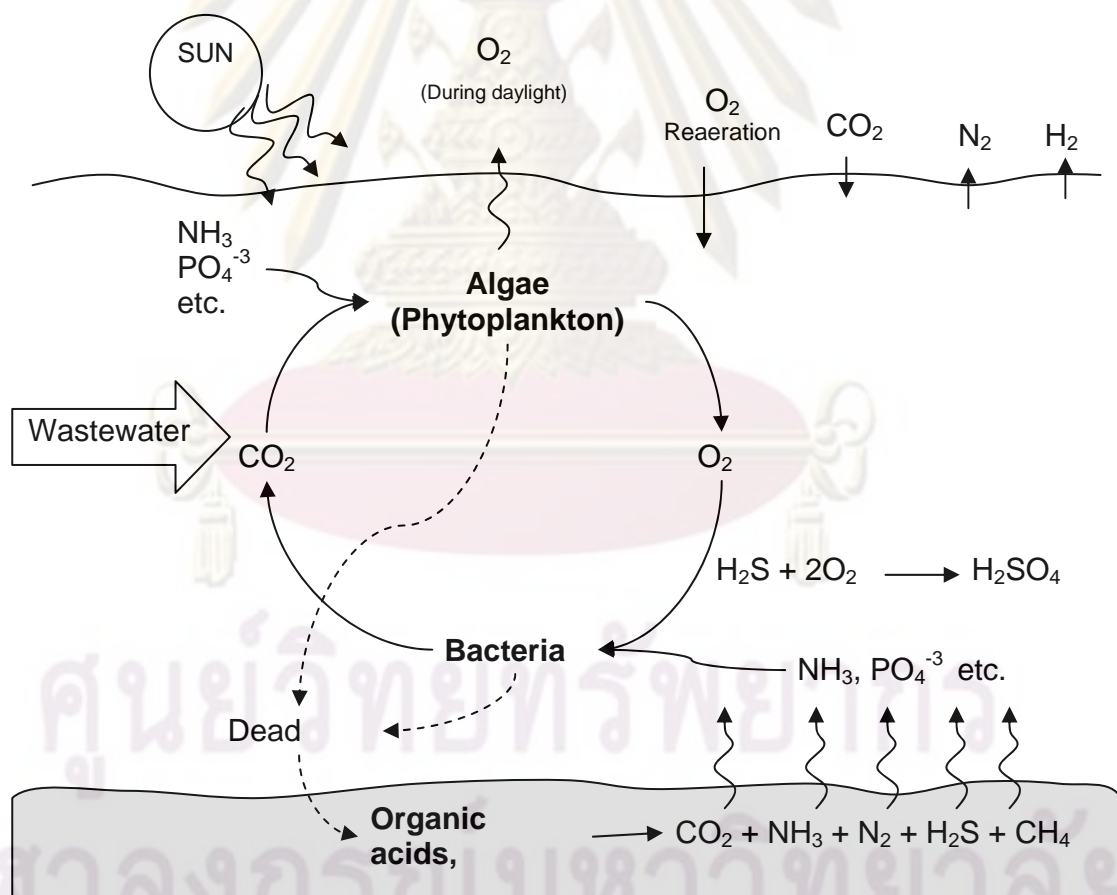
Figure 2.2 Biological relationships between phytoplankton and bacteria in earthen stabilization pond. (Nootong, 2008)

2.5.2.2 Biofloc technology

Biofloc technology is the nitrogenous wastewater treatment and protein utilization strategy that is applicable in both freshwater and marine environments (Azim et al., 2008). This method was sustainable in both intensive and extensive aquaculture production (Hari et al., 2006; Avnimelech, 2006; Crab et al., 2007). Research works on biofloc technology so far have focused mainly on cultivation of shrimp and Tilapia. According to figure 2.3, the removal of inorganic nitrogen compounds (*e.g.*, NH_3, NH_4^+, NO_2^-) in biofloc technology systems is engineered based on an enhancement of heterotrophic bacterial growth to assimilate nitrogen and incorporate it into new cellular proteins during microbial biomass synthesis. As bacteria in water flourish reaching the density as high as 10^7 CFU, they tend to form noticeable amorphous aggregates (*i.e.*, biofloc), which are made up mostly from heterotrophic bacterial communities (Boyd and Clay, 2002; Burford et al., 2003). Other components namely autotrophic bacteria, microalgae, zooplankton, protozoa, inorganic matters (*e.g.*, sand) and remains of microorganisms are also presence (Burford et al., 2003; Avnimelech, 2007). Structures of biofloc appear irregular and highly-opened with the porosity ranged from 65 to 75% of the total aggregate volume (Avnimelech, 2006; Nootong and Shieh, 2008). Cruz and Ridha (1995) described the similarities in structures between biofloc in aquaculture systems and those normally found in activated sludge processes. Sizes of biofloc, ranging from 0.1 to 2.0 mm in diameter, are beneficial for bacteria living inside because predation from larger natural enemies such as protozoa and rotifers could be avoided (Verstraete et al., 2007). Living and nonliving components in biofloc are loosely-held together by inter-particle bonds and bacterial excretions called extracellular polymers (EPS), which are highly hydrated, charged biofilm matrixes comprised mainly from polysaccharides and proteins (Bache et al., 1997; Flemming and Wingender, 2001).
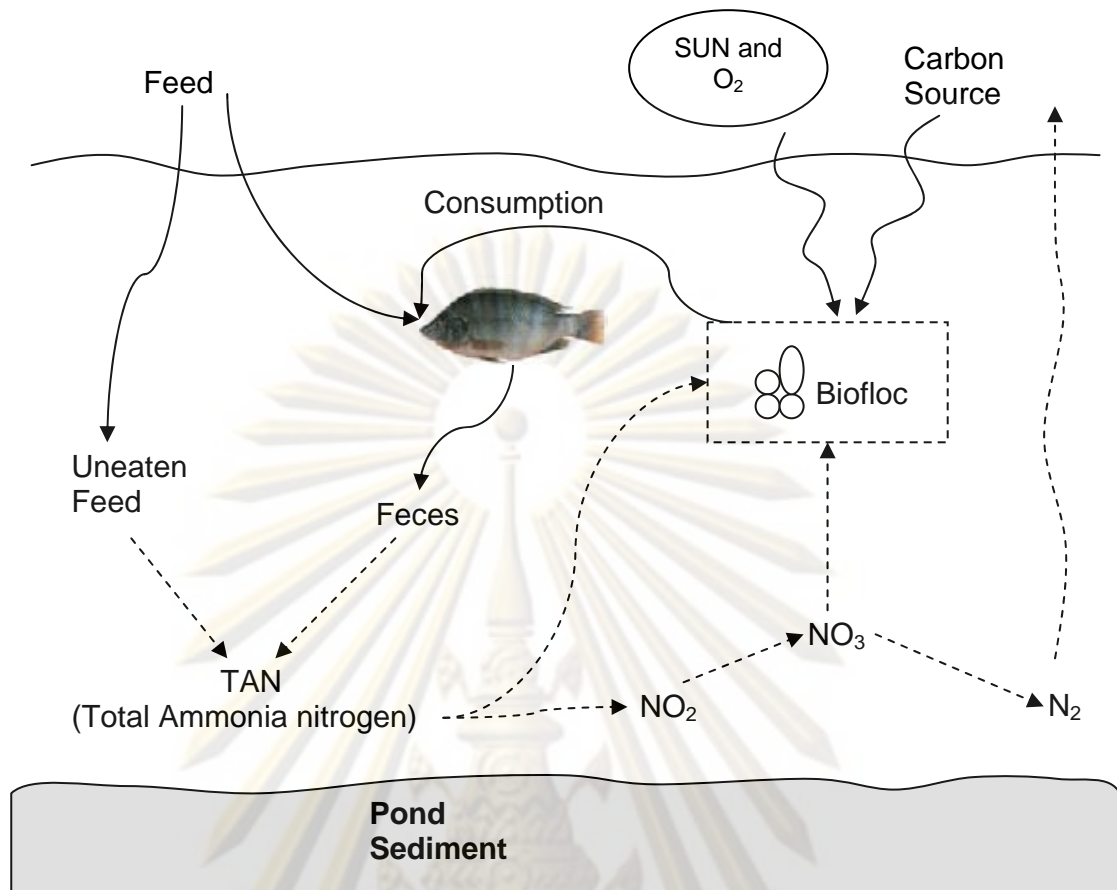
Figure 2.3 Water treatment and protein reutilization concept in biofloc technology system. (Nootong, 2008)

2.5.2.3 Engineering of biofloc technology

Equation (2.6) indicates the possible reaction pathway of inorganic nitrogen assimilation into new microbial biomass (*i.e*., $C_5H_7O_2N$) by heterotrophic bacteria. This process, if properly adjusted to enhance microbial nitrogen uptake, could effectively reduce excessive ammonia and nitrite accumulations in water. According to equation (2.6), the following factors can influence successful startup and the maintenance of biofloc technology systems.

$$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \quad \rightarrow$$

$$C_5H_7O_2N + 6.06H_2O + 3.07CO_2 \qquad (2.6)$$
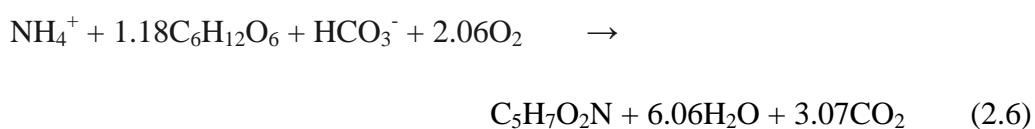
1. Addition of organic carbon

Transition of ponds to more heterotrophic dominance could be accomplished and maintained by providing sufficient amounts of organic carbon (*i.e.*, $C_6H_{12}O_6$) or other forms of carbohydrate (*e.g.*, molasses, starch, cassava meal). Organic carbon compounds are required as food and energy sources for heterotrophic bacteria during new biomass generation. Tacon et al. (2002) reported a gradual transformation of outdoor shrimp cultivating tanks from phytoplankton based autotrophic food web towards bacterial based heterotrophic food web after 4 weeks of the daily addition of shrimp diets containing wheat as a major ingredient. In another report, addition of grain feeds (*i.e.*, mixtures of soybean, wheat grain and corn) containing 18 to 22% proteins and molasses into high-density (120 shrimp/m^2) zero-exchanged shrimp ponds in Belize could promote the change of pond dynamics from phytoplankton to more heterotrophic dominance after 8 to 10 weeks of continued supplying organic-mixed shrimp feeds (Boyd and Clay, 2002; Burford and Lorenzen, 2004).

Excessive accumulation of inorganic nitrogen compounds in biofloc technology systems could be avoided by maintaining high substrate C:N ratios within ponds under aerobic condition (Avnimelech et al., 1994; McIntosh, 2001). Avnimelech (1999) described a complete removal of 10 mg NH_4^+-N/L within the period of 2 hours following the addition of glucose into suspension at the concentration 20 times higher than that of total ammonia nitrogen (TAN). In another work, a significant reduction of ammonia was also observed when the shrimp cultivating tank was supplied with glucose and cassava meal, which were added daily at the quantity 7 times higher than the assumed TAN excretion rate of 33% (Avnimelech et al., 1994). Recent work by Fontenot et al. (2007) used molasses and ammonium salts to adjust C:N ratios of shrimp aquaculture wastewater and obtained the result that the optimal C:N ratio of 10:1 was for the most effective for wastewater treatment. Similar results were reported by Azim et al. (2008) such that the optimal biofloc development measured in term of volatile suspended solids (VSS) and BOD$_5$ was observed at the C:N ratio of 11.6. At this optimal condition, the corresponding biofloc production rates ranged from 3 to 5 g C/m^3/day. Avnimelech (1999) had done the work separately on biofloc technology and suggested the systematic method for quantifying the amount of organic carbon needed to supply biofloc technology ponds in order to remove inorganic nitrogen compounds in water effectively. For instance,

the amount of organic matters required to removal 1 mg NH_4^+-N/L could be calculated by following this simple approach (Avnimelech, 1999).

(1) The amounts of organic carbon assimilated by heterotrophic bacteria (ΔC_{mic}) were estimated using $\Delta C_{mic} = (\Delta CH)(E)(C\%)$, where ΔCH is the amount of added carbohydrate, which is required to assimilated nitrogen; E is the microbial conversion efficiency, which is the ability of bacteria to metabolize added organic carbon, generally ranged from 40 to 60%; and C% is the carbon content in added carbohydrate normally found at 50% for most substrates.

(2) The amount of nitrogen required to produce new cell materials would depend on bacterial C:N ratios, and could be written as: $\Delta N = \Delta C_{mic}/[C:N]_{mic} = (\Delta CH)(\%C)(E)/[C:N]_{mic}$, where ΔN is the amount of nitrogen assimilated for the production of new cell materials; and $[C:N]_{mic}$ is the C:N ratio of bacteria.

(3) By employing approximate values of %C, E and $[C:N]_{mic}$ as 0.5, 0.4 and 4, respectively (Gaudy and Gaudy, 1980; Avnimelech, 1999), the amounts of carbohydrates that are required to sequester 1.0 g of NH_4^+-N is 20 g.

2. Aeration and mixing

Biofloc technology ponds could be considered as biological completely mixed reactors. Under sufficient oxygenation and mixing, heterotrophic bacteria are able to assimilate as much as 40 to 60% of the total organic matters added. Inadequate aeration and mixing result in excessive organic loading in water and quick solid sedimentation at bottom of the pond that could easily develop into anaerobic conditions (Avnimelech, 1999; and Avnimelech and Ritvo, 2003). As a result, aeration is normally provided 24 hours a day and is typically achieved via mechanical aeration devices to maintain DO concentrations above 4.0 mg O_2/L (Boyd and Clay, 2002; Avnimelech and van Wyk, 2007). Malfunction of aeration equipments in

biofloc technology systems could lead to a rapid decrease of oxygen inventory to reach critical levels within 3 hours (Vanitchanai and Nootong, 2008). The availability of dissolved oxygen in water may also determine biofloc structures. Filamentous bacteria tends to dominate at DO < 2 mg O_2/L (Martin et al., 2004; Tchobanoglous, et al., 2004), whereas larger and more compact bacteria (*i.e.*, floc forming) are more abundant at higher DO levels (DO = 2.0 – 5.0 mg O_2/L) (Wilen and Balmer, 1999; Martin et al., 2004). Schryver and Verstratete (2008) recommended operating biofloc technology ponds at high DO levels to produce the floc volume index (FVI) greater than 200 mL/g to avoid biofloc settling too quickly in the dead zone region of ponds.

Providing excessive physical forces from aeration and mixing may breakup and hinder biofloc development (Sales and Shieh, 2006). The average biofloc size decreases when higher mixing intensity (*i.e.*, higher shear force) is applied. For example, Bigg and Lant (2000) demonstrated in activated sludge system that stable floc size was approximately 130 μm when the velocity gradient was fixed at 19.4 s^{-1}, whereas the average floc size decreased to only 20 μm at the applied velocity gradient of 346 s^{-1}. Previous experiments recommended operating mechanical devices to provide optimal fluid shear rates in the range between 10 and 100 s^{-1} for intensive aquaculture cultivation (Boyd and Tucker, 1998; McGraw et al., 2001; Schuur, 2003). This is corresponding to the power input from 1 to 10 W/m^3 (30 W/m^3 for mixing in activated sludge process). Under the moderate mixing rates, microbial cells are able to attain higher substrate uptake rates, flocculate and form permeable biofloc that would benefit from convective flow within ponds, and thus are able to grow at faster rates than a single cell in suspension (Logan and Hunt, 1988; Crab et al., 2007). Operation of mechanical aeration devices to produce shear rates beyond this range (*i.e.*, > 100 s^{-1}) tended to breakup biofloc aggregates (Crab et al., 2007).

3. Temperature

Temperature could influence the availability of dissolved oxygen in water particularly for the case of outdoor aquaculture cultivation where the difference between maximum and minimum temperature could be nontrivial. Oxygen is less soluble in water at higher temperature, for instance at the atmospheric DO concentration was reported at 9.08 mg/L at 20 °C, whereas it is only 6.93 mg/L at 35

°C (Tchobanoglous et al., 2004). The optimum temperatures for bacterial activity are in the range from 25 to 35 °C. Krishna and van Loosdrecht (1999) reported the occurrence of activated sludge bulking when the temperature was from 30 to 35 °C, and also suggested the temperature range between 20 and 25 °C to produce good sludge property with FVI about 200 mL/g.

4. Other chemicals

Certain chemicals such as short-chain activated silica can promote biofloc formation by serving as attached sites for bacteria. Addition of chemical-aided floc forming materials is well-established technique in domestic and industrial wastewater treatments, yet this practice in aquaculture cultivations remains limited. Only Boyd and Clay (2002) employed the addition of sodium silicate byproduct of zeolite manufacturing to improve aggregation of microbial floc in the zero-exchange shrimp cultivation ponds but did not elaborate the details.

2.5.3 Water characteristics of biofloc technology

Water characteristics of biofloc technology systems are different during the initial startup and long term operation due to varied microbial dominances in ponds. The NH_4^+-N concentrations usually decrease during the initial stage due to high nitrogen assimilating rates by heterotrophic bacteria and microalgae. A continued addition of organic carbon will promote the growth of heterotrophic populations that help maintaining NH_4^+-N concentrations at low levels. NO_2^--N and NO_3^--N stabilize at very low concentrations because they are not products of assimilating process (i.e., equation 2.1), whereas large quantities of suspended solids (SS) and volatile suspended solids (VSS) in water increase significantly. Once biofloc technology systems reached steady state, NH_4^+-N will be relatively constant at acceptable moderate levels, while NO_2^--N and NO_3^--N will be detected at minimal concentrations. The amounts of VSS will continue to increase at appreciable rates while other parameters such pH and alkalinity will remain relatively constant. In addition, water of biofloc technology systems tends to be turbid due to high biomass yield at 8.07 g VSS/g N. This amount of VSS production is approximately 20 times

higher than the solid generation rates by autotrophic nitrification process (Ebeling and Timmons, 2007).

Other nitrogen controlled mechanisms can proceed simultaneously within biofloc technology ponds as well. For instance, autotrophic nitrification was observed to take place in high-rate shrimp and Tilapia production systems containing flocculated matters when the total ammonia within water column was about 1 to 2 mg N/L or higher (Boyd and Clay, 2002; Brune et al., 2003). Because of nitrification that releases hydrogen ions in water, alkalinity of biofloc technology ponds would continue to decrease unless some forms of alkalinity such as limestone was supplied. Overall nitrifying rates were likely to be limited due to faster growth of heterotrophic populations (Brune et al., 2003). Only 15% of inputs NH_4^+-N were converted into nitrate while significant fractions at 43% were subjected to heterotrophic bacterial uptake (McIntosh, 2001). Higher percentages between 29 and 43% of nitrogen conversion by autotrophic nitrification could be achieved in heavier loads, thoroughly mixed, well-aerated Tilapia cultivation systems (Avnimelech et al., 1994). However, this system was not considered an efficient nitrification because it was characterized by high BOD/NH_3-N ratios. Nitrifying populations were likely to be less than 5% of total microbial biomass (Tchobanoglous et al., 2004).

2.5.3.1 Feed reutilization

An important feature of biofloc technology, which offers the distinct advantage over traditional aquaculture cultivations and attached-growth external biofilters systems, is the reduction in feed expenses due to more effective protein recovery from uneaten feed via biofloc consumption by aquacultures. Aquatic animals in biofloc technology systems are able to consume proteins in feeds at least twice; once in feed and later from biofloc proteins. This would reflect into substantial increases of protein recovery from about 25 to 30% in conventional cultivation to almost 50% in commercial farms adopting biofloc technology concept. Aquaculture cultivating system employing biofloc technology could reduce the feed expense by either lowering the amount of feed required or switching the feeds from high to low protein contents. For example, Avnimelech et al. (2004) cultivated Tilapia in freshwater by using sorghum as supplemental carbon source in combination with feed

pellets containing only 20% proteins. The experimental outcome indicated a significant increase of protein recovery rates from 23 to 43% that was equivalent to almost 50% cost saving when using 30% protein feed pellets alone. McIntosh (2001) similarly reported that the feed expense of Tilapia farming was lowered by \$0.2 per kg of harvested fish because of the transition from 30% to 20% protein feeds. Additional results by Avnimelech (2006) found that Tilapia reared in biofloc technology systems containing biofloc volume about 20 to 30 mL/L did not actively jump toward feeds unlike that reared in traditional ponds. This observation could be explained due to the fact that fish were continuously eating biofloc between meals. Digestive track analysis between meals confirmed the presence of detritus in abundance. Recently, nitrogen uptake of microbial biofloc by Tilapia was estimated at 0.25 g N/kg protein, which is equivalent to the daily uptake of 6.2 g N/kg dry biofloc (Avnimelech, 2007). The uptake rate obtained was only 60% of the rate computed by simplified mass balance calculation, which neglected microbial degradation within biofloc. For saline cultivating systems, nitrogen recovery rates were comparable to those in freshwater. Nitrogen recovery rates in super-intensive shrimp cultivation in Belize were roughly 39% of the quantity available in feeds (Boyd and Clay, 2002); that was almost double the values typically reported in traditional shrimp cultivation with frequent water exchange (Boyd and Tucker, 1998 cited in Boyd and Clay, 2002). An experiment by Panjaitan (2004) indicated as high as 70% reduction of feed requirement when biofloc technology was employed instead of traditional opened-pond systems. Additional work by Hari et al. (2004) utilized biofloc technology by adding 0.39 kg of tapioca flour as supplemental carbon source per kg of 25% protein shrimp diets during extensive in extensive shrimp (*Penaeus monodon*) production. Cultivating results demonstrated that 35% reduction in feed expense and 54% increase in revenue could be accomplished compared to supplying shrimps with only 40% protein feed pellets. According to results obtained, a sustainable shrimp production by biofloc technology could be achieved in grow-out extensive ponds because (1) lower protein dietaries could be used (*i.e.*, 40% to 25%) without compromising shrimp survival rates; (2) concentrations of inorganic nitrogen compounds in production ponds were within limitation and (3) reduction in nutrient discharge was met (Hari et al., 2004; 2006).

2.5.3.2 Nutrition and probiotic effect

Raw materials used to produce 1.0 kg of aquaculture feeds normally comes from fishmeal and fish oil, which are produced from catching 1 to 5 kg of fish from oceans (Naylor et al., 2000). This practice represents non-sustainable way of producing feeds that could be partially solved by adopting biofloc technology concept. Decamp et al. (2003) and Defoirdt et al. (2007) indicated the presence of vitamins, trace minerals, and poly-β-hydroxylbutyrate (PHB), which is a storage polymer molecule that helps preventing a pathogenic infection in aquacultures. Further analysis of biofloc samples by Azim et al. (2008) indicated the proximate composition of biofloc to contain slightly higher than 50% of crude proteins, 4% fiber, 7% ash and 22 kJ/g on the basis of dried weight. This proximate composition is considered appropriate for feeding herbivorous/omnivorous species such as carp and Tilapia.

Biofloc also exhibit probiotic effects on aquacultures as suggested by the results presented by Avnimelech and Bejerano (2007). In this work, a dense *Streptococcus iniae*, a common bacterial strain causing unmarketable appearance and heavy mortality for fish, was injected to 10% of Tilapia population reared in conventional and biofloc technology ponds. There was no significant difference regarding infection of *Streptococcus* between the injected fish in both ponds, whereas for non-injected fish the rate of disease infections were as much as 25% lower in biofloc technology ponds. It was postulated that dense heterotrophic populations ($\approx$ $10^6 - 10^7$ per mL) in biofloc ponds attacked pathogens released from sick and dying fish from infecting remaining healthy fish populations. Additional work by Moss et al. (2001) suggested positive impacts of consuming biofloc to shrimp digestive enzymes and gut microflora, while Taoka et al. (2006) reported enhanced immune parameters in Tilapia, resulting in higher resistance to *Edwardsiella tarda* infection.

2.5.3.3 Disadvantages

Biofloc technology also has several disadvantages. Due to high heterotrophic growth, significant amounts of suspended solids are produced during aquaculture production causing high turbidity of water in ponds that could become a problem to

some aquatic species. High sludge production also accompanies by significant CO_2 formation that leads to a rapid pH reduction in production ponds. Excessive sludge generation and maintaining of high sludge age could enhance the proliferation of protozoans, which are known natural predators of heterotrophic microorganisms. The presence of protozoans at high numbers could reduce heterotrophic populations that directly affect the capability of inorganic nitrogen uptake in biofloc technology ponds (Avnimelech and van Wyk, 2007). Therefore, a weekly sludge draining must be performed as a mean to reduce a possible sludge accumulation on pond bottom and to avoid excessive turbidity in shrimp farms. More frequent operations as high as few times a day are possible in fish farms due to higher solid loadings (McIntosh, 2001; Boyd and Clay, 2002). Alkalinity in forms of $CaCO_3$ and $NaHCO_3$ is required to maintain optimal pH and alkalinity ranges between 7 and 8 and between 100 and 150 mg/L $CaCO_3$, respectively. Another limitation of biofloc technology is high oxygen and mixing requirement to ensure that heterotrophic bacteria are suspended in water and able to degrade added carbon aerobically. Oxygen demand in biofloc technology system is more intensive than conventional cultivation. Oxygen requirement was estimated in the range from 1.0 to 1.2 kg O_2 per kg feed (Avnimelech and van Wyk, 2007). Despite intensive oxygen requirement, cost of oxygenation in biofloc technology systems is likely to be offset with pumping and aeration expenses required in external biofilters systems mediating nitrification and denitrification (Losordo and Westerman, 1994).

CHAPTER III

RESEARCH METHODOLOGY

This research can be divided into two main sections: the first section involved the determination of optimal conditions for biofloc formation without fish culture while the second section applied the data obtained earlier to the zero-water exchanged tilapia cultivation in biofloc systems. Figure 3.1 illustrates the flow diagram describing the experiment structure.

```
                        ┌───────────────────────────┐
                        │  Experimental Framework   │
                        └───────────────────────────┘
```
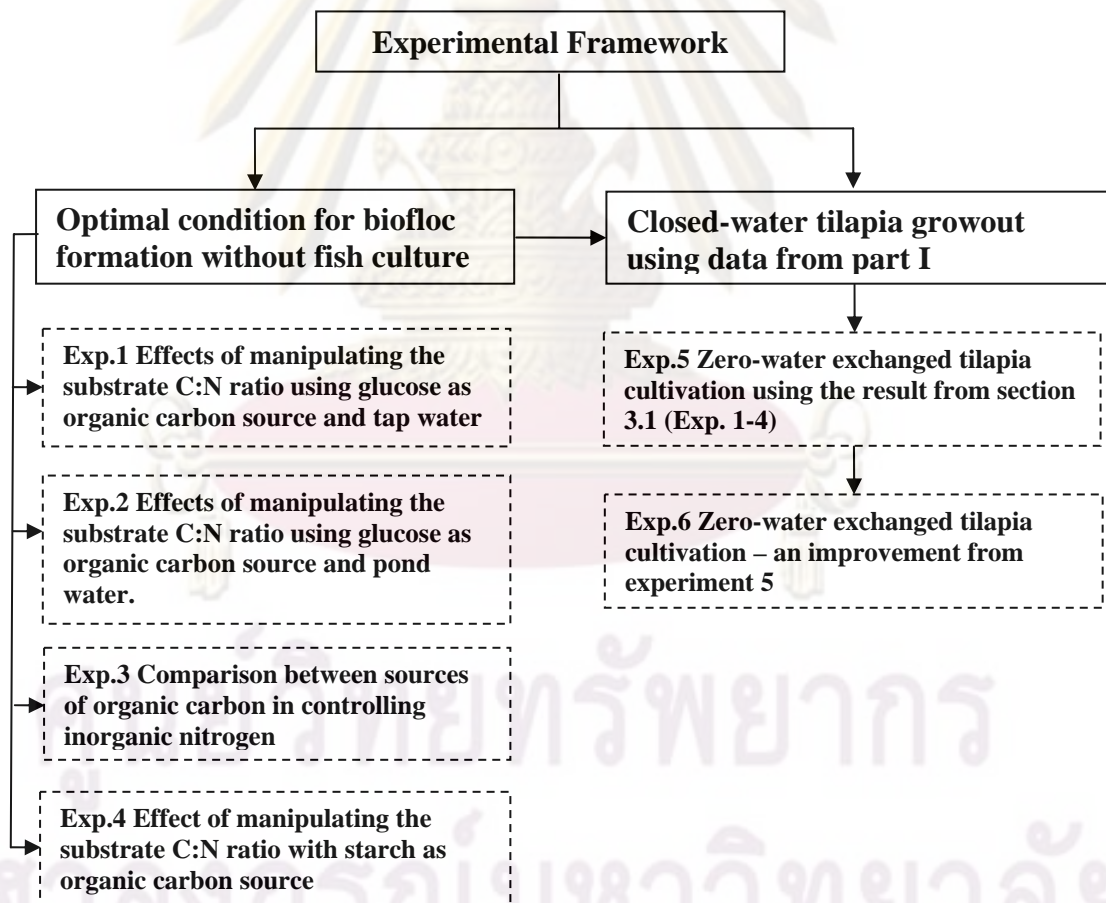

Figure 3.1 Framework of the experiments involved in this study

3.1 Optimal condition for biofloc formation without aquacultures

3.1.1 Experiment 1: preliminary information and the effects of manipulating the substrate C:N ratio using glucose as organic carbon source and tap water.

The experiment was carried out in one control (with 3 replications) and 4 treatments (with 3 replications) by using 15 identical glass bottles (7 L), which were filled up with tap water to attain the working volume of 4 L. Glucose and ammonium chloride (NH_4Cl) were used as carbon and nitrogen sources, respectively. Different amounts of glucose and ammonium chloride were added daily into each glass bottle to provide different sets of substrate C:N ratios. The control (Bottle B1, B2 and B3) was supplied daily with 15.3 mg NH_4Cl only to achieve the ammonium dose at 1.0 mg N/L. Different amounts of glucose having the weight of 20, 40, 80 and 160 mg were respectively added into the treatments on the daily basis to produce the substrate C:N ratios of 2:1, 4:1, 8:1 and 16:1. One diffusive stone aerator was installed in each glass bottle to provide adequate mixing and dissolved oxygen (DO) at greater than 3.0 mg O_2/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by regular addition of $NaHCO_3$. The glass bottles were located outdoor adjacent to the laboratory building to receive sunlight and were entirely covered by plastic lid to prevent rainwater penetration. Daily grasp samples of water from each bottle were obtained and immediately analyzed for total ammonia nitrogen (TAN), nitrite (NO_2^--N) and nitrate (NO_3^--N) concentrations according to APHA (1998). Details of the analysis can be found in Appendix A (subsection A-2, A-3 and A-4).

3.1.2 Experiment 2: biofloc formation: the effects of manipulating the substrate C:N ratio using glucose as organic carbon source and pond water.

The experiment was carried out in one control (with 3 replications) and 2 treatments (with 3 replications) by using 9 identical glass bottles (7 L), which were filled up with water obtained from natural pond located near the Faculty of Science at Chulalongkorn University, to attain the working volume of 4 L. Glucose and

ammonium chloride (NH_4Cl) were used as the carbon and nitrogen sources, respectively. Different amounts of glucose and ammonium chloride were added daily into each glass to provide different sets of substrate C:N ratios. The controls (Bottle B1, B2 and B3) were supplied daily with 15.3 mg NH_4Cl only to achieve the ammonium dose at 1.0 mg N/L. Treatment 1 (Bottle B4, B5 and B6) was supplied daily with 15.3 mg NH_4Cl and 20 mg of glucose to achieve the daily substrate C:N ratio addition at 2:1. Treatment 2 (Bottle B7, B8 and B9) was added on the daily basis with 15.3 mg NH_4Cl and 160 mg of glucose to attain the daily substrate C:N ratio addition at 16:1. One diffusive stone aerator was installed in each glass bottle to provide adequate mixing and DO at greater than 3.0 mg O_2/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by regular addition of $NaHCO_3$. Glass bottles were located outdoor adjacent to the laboratory building to receive sunlight and were entirely covered by plastic lids to prevent rainwater penetration. Daily grasp samples of water from each glass bottle were obtained and immediately analyzed for TAN, nitrite, nitrate and suspended solids according to APHA (1998). Chlorophyll was measured according to Strickland and Parson (1972). Details of the analysis can be found in Appendix A (subsection A-2, A-3, A-4, A-6 and A-7).

3.1.3 Experiment 3: comparison between sources of organic carbon in controlling inorganic nitrogen

The experiment was carried out in 1 control (with 3 replications) and two treatments (with 3 replications) by using 9 identical glass bottles (7 L), which were filled up with tap water to attain the working volume of 4 L. Glucose and tapioca starch (Fish Brand) were used as carbon source while ammonium chloride remained as the nitrogen source. The control (Bottle B1, B2 and B3) was supplied daily with 30.57 mg NH_4Cl only to attain the ammonium dose at 2.0 mg N/L. Treatment 1 (Bottle B4, B5 and B6) was supplied on a daily basis with 30.57 mg NH_4Cl and 320 mg of glucose to achieve the substrate addition at the C:N ratio of 16:1. Similarly, treatment 2 (Bottle B7, B8 and B9) was provided with 30.57 mg NH_4Cl and 512 mg of starch to attain the daily substrate addition at the C:N ratio of 16:1. One diffusive stone aerator was installed in each glass bottle to provide adequate mixing and DO at

greater than 3.0 mg O_2/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by regular addition of $NaHCO_3$. The glass bottles were located outdoor adjacent to the laboratory building to receive sunlight and were entirely covered by plastic lids to prevent rainwater penetration. Daily grasp water samples were obtained from each glass bottle and immediately analyzed for TAN, nitrite and nitrate according to APHA (1998). Details of the analysis can be found in Appendix A (subsection A-2, A-3 and A-4).

3.1.4 Experiment 4: effect of manipulating the substrate C:N ratio with tapioca starch as organic carbon source

The experiment was carried out in 1 control (with 3 replications) and two treatments (with 3 replications) by using 9 identical glass bottles (7 L), which were filled up with tap water to attain the working volume of 4 L. Tapioca starch (Fish Brand) was used as a sole carbon source while ammonium chloride and commercial shrimp diets were employed as the combined nitrogen sources. The proportion of nitrogen mass from ammonium chloride and shrimp diets was fixed at 4:1 (i.e., 1.0 g of nitrogen mass was from 0.8 g N from NH_4Cl and 0.2 g N from shrimp diets). The control (Bottle B1, B2 and B3) was supplied daily with 15.3 mg NH_4Cl and 17.7 g of 20% shrimp diets to attain ammonium dose at 1.0 mg N/L. Treatment 1 (Bottle B4, B5 and B6) were supplied on a daily basis with 15.3 mg NH_4Cl and 17.7 g of 20% shrimp diets and 66 mg of starch to achieve the substrate addition at the C:N ratio of 2:1. Similarly, treatment 2 (Bottle B7, B8 and B9) was provided with 15.3 mg NH_4Cl and 17.7 g of 20% shrimp diets and 528 mg of tapioca starch to attain the daily substrate addition at the C:N ratio of 16:1. A diffusive stone aerator was installed in each glass bottle to provide adequate mixing and DO at greater than 3.0 mg O_2/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by regular addition of $NaHCO_3$. The glass bottles were located outdoor adjacent to the laboratory building to receive sunlight and were entirely covered by plastic lids to prevent rainwater penetration. Daily grasp water samples were obtained from each glass bottle and immediately analyzed for TAN, nitrite and nitrate according to APHA (1998). Details of the analysis can be found in Appendix A (subsection A-2, A-3 and A-4).
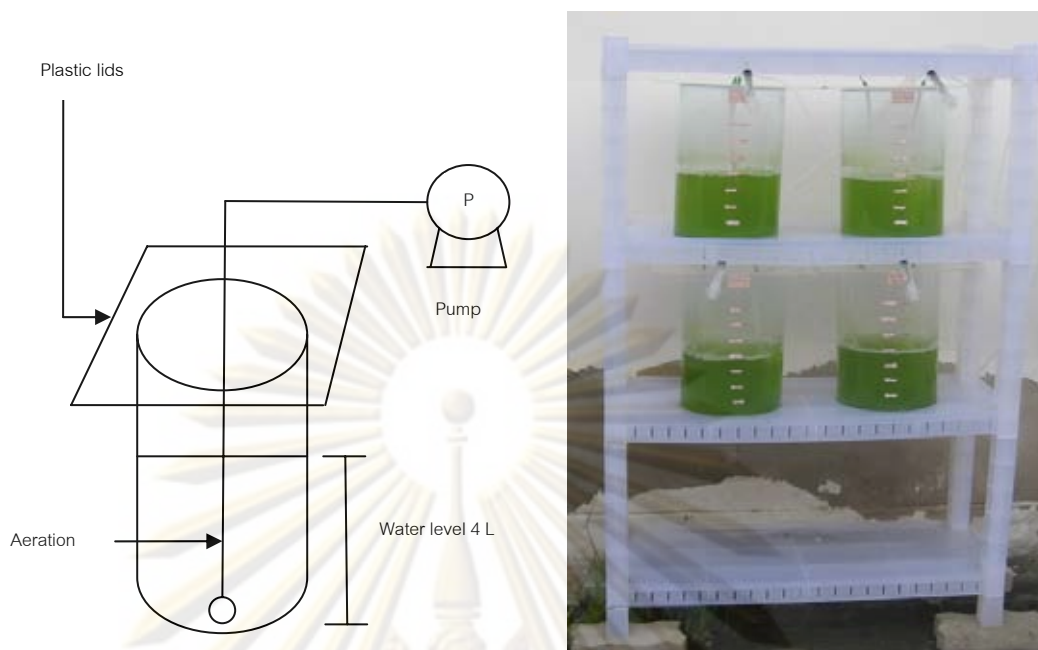
Figure 3.2 (Left) Schematic of experiment (Right) Actual picture of the experiment to determine the optimal C:N ratio for biofloc formation without the presence of fish culture (Part I.).

3.2 The closed-water biofloc system for Tilapia cultivation using result from Part I

3.2.1 Experiment 5: zero-water exchanged Tilapia cultivation using the result from section 3.1.

The experimental result from section 3.1 was used in this section to carry out the zero-water exchanged Tilapia cultivation in biofloc systems. Nine replicated fiber-glass containers (130 L) were filled with tap water to attain a working volume 130 L. Continuous aeration of filled tap water in the containers was carried out for several days to remove residue Chlorine. Four diffusive stone aerators were placed in each container to maintained well-mixed condition and the DO at greater than 4 mg

O_2/L. All fiber-glass tanks were covered with transparent plastic sheets to prevent rainwater and partially allowed sunlight.

The zero-water exchanged Tilapia cultivation was carried out in the described containers for 45 days. Nile Tilapia (*Oreochromis niloticus*) with an average weight of 30 ± 4.5 g was stocked in each container to obtain an initial weight density at about 3.0 kg/m^3. Tilapia was fed with 30% protein commercial feeds (N-source) at 3% of total fish weight per day. The controls (with 3 replications) were supplied on the daily basis with 30% protein feeds only. Treatment 1 (with 3 replications) was provided daily with Tilapia feeds and tapioca starch (C-source) at the weighted C:N ratio of 2:1. Treatment 2 (with 3 replications) was also provided daily with the Tilapia feeds and tapioca starch (C-source) but increased the weighted C:N ratio to 16:1. Thus, the actual amount of tapioca starch varied according to the quantity of Tilapia feeds added into the containers. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L $CaCO_3$, respectively by adding $NaHCO_3$. The daily grasp samples of water from each tank was obtained and immediately analyzed for TAN, nitrite, nitrate and total suspended solids (SS) according to APHA (1998). Microscopic examination of biofloc samples by fluorescent microscopy was performed according to Avnimelech, et al. (2007) and Azim et al. (2008). The biofloc volume was determined by the 30 minute sedimentation in an Imhoff cone. At the end of experiment, the entire tilapia in each tank was caught to determine its weight and length. The obtained information was used to determine the average daily growth (ADG), feed conversion ratio (FCR) and survival rate.

3.2.2 Experiment 6: Zero-water exchanged Tilapia cultivation – an improvement from experiment 5.

Since the size of containers in experiment 5 was found too small for Tilapia cultivation, larger tanks were used instead. Four replicated fiber-glass tanks (500 L), which were filled with tap water to attain a working volume 500 L, were placed outside the laboratory building. An initial suspended solid concentration of the pond water was determined at 25 mg SS/L. Four diffusive stone aerators and a submerged pump were placed in each tank to maintain well-mixed condition and DO at greater

than 4.0 mg O_2/L. All fiber-glass tanks were covered with the semi-transparent plastic sheets to prevent rainwater and partially allow sunlight. The zero-water exchanged Tilapia growout was carried out in the described fiber-glass tanks for 60 days. Nile tilapia (*Oreochromis niloticus*) with an average weight of 30 ± 4.5 g was stocked in each tank to obtain the initial weight density at about 3.0 kg/m^3. Tilapia was fed with 30% protein commercial feeds (i.e., N-source) at 3% of total fish weight per day. Tank 1 and 2 (T1 and T2) were replicated control systems, which were supplied on a daily basis with 30% protein feeds only. Tank 3 and 4 (T3 and T4) were considered as the treatments and were provided daily with 30% protein Tilapia feeds and tapioca starch fish brand (C-source) at the weighted C:N ratio of 16:1. Thus, the actual amount of tapioca starch varied during the experiment according to the quantity of tilapia feeds added into the tanks. The pH and alkalinity were respectively maintained between 7 and 8 and between 100 and 150 mg/L $CaCO_3$ by adding $NaHCO_3$. Diffusive stone aerators and submerge pump were employed to maintain well-mixed condition and DO > 4.0 mg O_2/L for each tank. Tilapia cultivation experiment was carried out without any solid removal.

Daily grasp samples of production water from each tank was obtained and immediately analyzed for TAN, nitrite, nitrate and total suspended solids (SS) according to APHA (1998). Details of inorganic nitrogen analysis can be found in Appendix A (subsection A-2, A-3 and A-4). The biofloc volume was determined by the 30 minute sedimentation in an Imhoff cone. Water samples from each of the control systems (T1 and T2) were mixed together, filtered through the Whatmann glass and allowed to dry overnight. These samples were sent to the Department of Chemistry at the Mahidol University to determine the proximate analysis for the C, H and N contents (CHNS/O Analysis, 2400 Series II Perkin Elmer Company). Same procedure was also applied for samples from the treatments. Mixed suspended solid samples from the controls and treatments were allowed to settle, drain by peristaltic pump and dry overnight before undergoing the PCR-DGGE analysis to determine the microbial diversity according to Muyzer et al. (1993). Details of the PCR-DGGE analysis can be found in Appendix B. The statistically analysis (t-test) between the controls and treatments was carried out by using Microsoft Excel 2007. For the nitrogen balance calculation, nitrogen in Tilapia biomass was calculated according to the following assumptions: Tilapia dried weight is approximately 33% of the total wet

weight and on average and Tilapia contained 45% protein (Wutikumpoln, 2003). Moreover, it was assumed that starch contained carbon at approximately 50% of the total weight (Avnimelech, 1999).

During the experiment, unfortunately the electrical failure occurred to cause a malfunction in aeration equipments. This caused a rapid decrease in oxygen concentration. Therefore, an independent experiment was set up to determine oxygen consumption rate. Approximately 1 L of suspended biofloc from the controls (2 replications) and treatments (2 replications) was completely filled up the empty space of 1 L glass bottle. Each bottle was tightly sealed to prevent air leakage and was wrapped with aluminum foil to prevent the growth of phytoplankton. Each glass bottle was equipped with two diffusive stone aerators to provide saturated DO concentration at about 8 mg O_2/L before starting the experiment. Aeration was switched off and the DO concentration was measured at every 15 minutes interval by using dissolved oxygen probe (HI 91410, Hanna; USA).
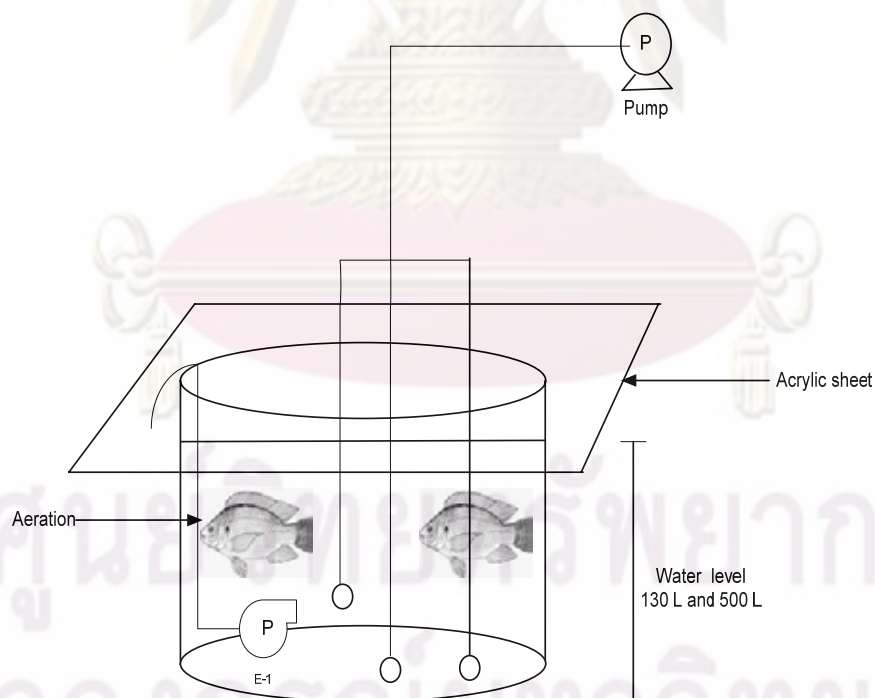
Figure 3.3 Schematic of the experimental system to cultivate tilapia by using the biofloc technology systems (Part II).

Table 3.1 Summary of analysis methods performed in the entire experiment

Parameter	Monitoring interval	Analytical Tool	Reference
DO (mg O_2/L)	Daily	DO meter method (HI91410, Hanna)	APHA (1998)
Temperature (^{o}C)	Daily	Logging pH meter (YSI, pH 10)	APHA (1998)
pH	Daily	pH meter method (YSI, pH 10)	APHA (1998)
TSS (mg/L)	Biweekly	Total solids dried at 103-105°C (Method 2540 D.)	APHA (1998)
TAN (mg/L)	Daily	Phenate method (Method 4500-NH_3 F)	APHA (1998)
NO_2^--N (mg/L)	Daily	Colorimetric method (Method 4500-NO_2^- B)	APHA (1998)
NO_3^--N (mg/L)	Daily	Ultraviolet spectrophotometric screening method	APHA (1998)
TN (mg/L)	Weekly	Ultraviolet spectrophotometric screening method	Gross et al. 1999
DTN (mg/L)	Weekly	Ultraviolet spectrophotometric screening method	Gross et al. 1999
Chlorophyll (mg/m^3)	Biweekly	Spectrophotometric determination of chlorophylls	Strickland and Parson 1972
Alkalinity (mg $CaCO_3$/L)	Biweekly	Titration method (Method 2320 B)	APHA (1998)

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The laboratory scale experiment without fish culture to determine the optimal substrates C:N ratio for biofloc formation

4.1.1 Experiment 1: effects of manipulating the substrate C:N ratio using glucose as organic carbon source and tap water.

Experiment 1 was carried out to obtain the preliminary result for biofloc formation. Since tap water contained very low amount of microorganisms, approximately 5 g of sediment from shrimp cultivating tank in the same laboratory were used as initial microbial seeding. Glucose was chosen as an initial carbon source since it is easy to obtain commercially and, more importantly, it is easily consumed by microorganisms. The source of nitrogen is ammonium chloride. As shown in Fig 4.1A, the daily addition of ammonium chloride at 1.0 mg N/L produced sequential buildups of TAN and nitrite that closely followed the characteristic of nitrifying system startup. The extent of TAN and nitrite accumulation was clearly related with the amount of glucose added daily. Initially, the TAN in each bottle increased from negligible levels (i.e., TAN < 0.1 mg N/L) to reach the maximum values ranging from 14.6 to 23.4 mg N/L before beginning to decline after day 6. Detailed examination revealed that an addition of glucose and ammonium chloride in treatment 4 (i.e., C:N = 16:1) was able to keep TAN at lower levels than the control and other treatments. Insignificant differences TAN were observable among the remaining substrate C:N ratios. For nitrite, the daily supplement of glucose and ammonium chloride in treatment 3 and 4 (i.e., C:N = 8:1 and 16:1) was capable of producing the nitrite concentration below 0.2 mg N/L for the entire experiment while the use of other substrate C:N ratios produced the maximum nitrite concentrations in the range from 0.96 to 1.75 mg N/L (Fig 4.1B). Based on the result presented, manipulating the extent of glucose addition was able to produce the different

inorganic nitrogen profiles in water. Maintaining high substrate C:N ratio at 16:1 seemed to offer the most promising result. This result concurred with the hypothesis made by Avnimelech (1999) that recommended using the high C:N ratio at 20:1 to remove TAN in water. According to Fig. 4.1C, the level of nitrate was relatively smaller than TAN. Nitrate appeared to increase steady for all bottles to suggest the occurrence of nitrification. The highest nitrate production was associated with the controls, which were supplied on the daily basis with ammonium chloride only. Lower nitrate concentrations were associated with the presence of glucose in water. Organic carbon compounds served as the food and energy sources for heterotrophic bacteria, which are known to possess higher oxygen affinity and able to grow faster than nitrifying bacteria (Sharma and Ahler, 1977). The fraction of nitrifying population was reported to increase with respect to decreasing organic carbon quantity (Tchobanoglous, et al., 2004). Due to limited growth rate of nitrifying bacteria, it was unlikely for nitrification to be the main pathway for inorganic nitrogen controls especially with the presence of organic carbon in water. In conclusion, experiment 1 demonstrated that high substrate C:N at 16:1 must be used in order to control the concentrations of inorganic nitrogen compounds and the occurrence of nitrification exerted a positive impact on water quality as it converted toxic ammonia into nitrate. In this experiment, only qualitative observation was made regarding the amount of biofloc formation: it appeared that treatment 4 (i.e., C:N = 16:1) had the highest turbidity due to formation of phytoplankton.
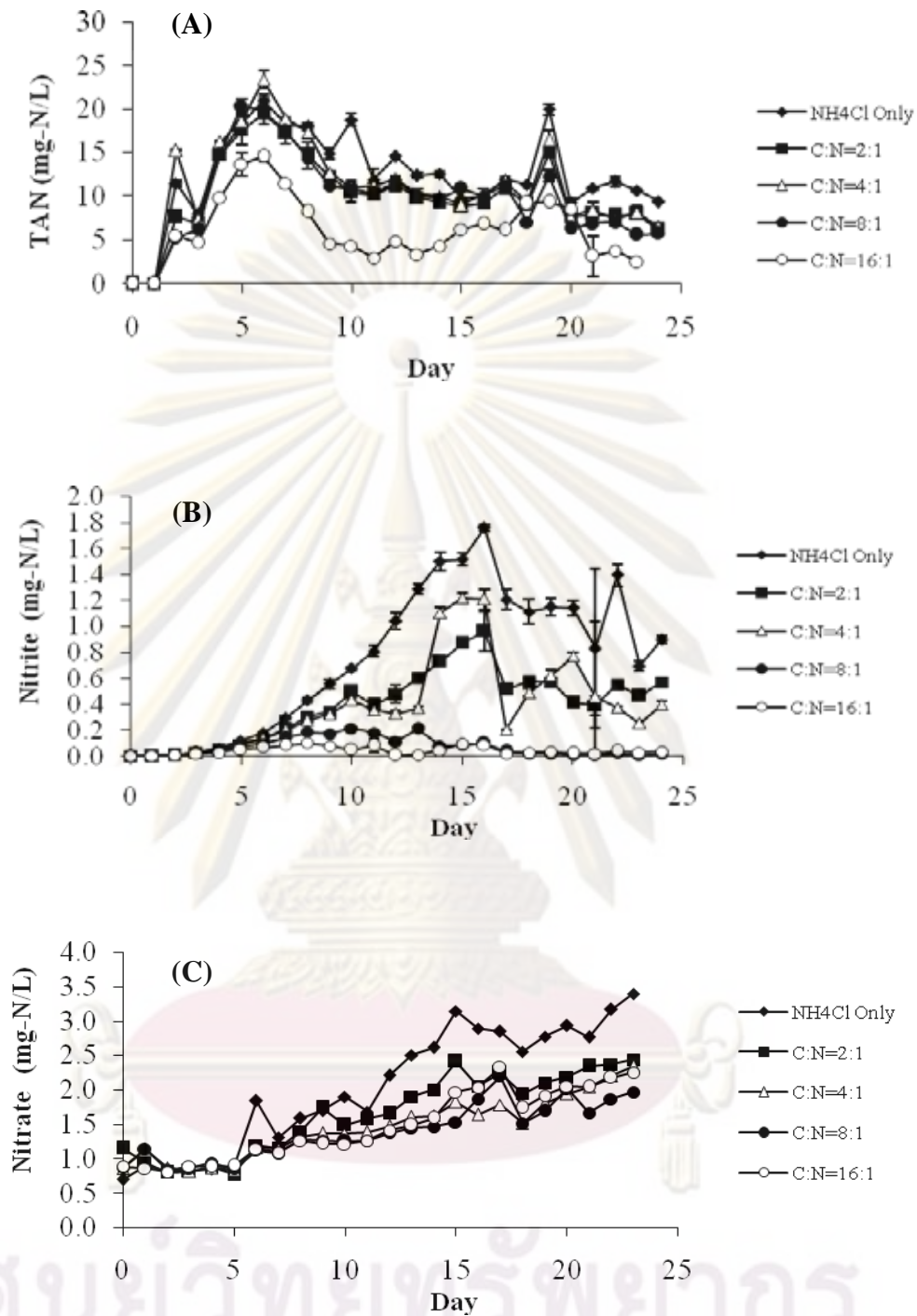
Figure 4.1 Effect of changing the substrate C:N ratio on TAN (A), nitrite (B), and nitrate (C) by manipulating the daily addition of glucose and ammonium chloride: (Control) NH₄Cl only, (Treatment 1) C:N = 2:1, (Treatment 2) C:N = 4:1, (Treatment 3) C:N = 8:1 and (Treatment 4) C:N = 16:1.

4.1.2 Experiment 2: effect of manipulating the substrate C:N ratio using glucose as organic carbon source and pond water in Chulalongkorn University as initial seeding.

Experiment 2 was carried out as an extension to experiment 1. In this experiment, however, natural water from a pond near the faculty of Science in the Chulalongkorn University was obtained to provide microbial seeding. Once again, daily addition of glucose and ammonium chloride were applied as the carbon and nitrogen sources, respectively. An initial suspended solid concentration from pond water was at 25 ± 5 mg SS/L. Without glucose supplement, TAN increased from less than 0.5 mg N/L to reach the maximum value at 8.0 mg N/L on day 9 before starting to decline slightly (Fig. 4.2A). Similar result was noticeable for treatment 1 (i.e., C:N = 2:1). In contrast, the daily provision of substrate in treatment 2 (i.e., C:N = 16:1) was more capable of maintaining TAN at less than 2.0 mg N/L for an entire experiment. For nitrite (Fig. 4.2B), the daily supplement of glucose and ammonium chloride at the C:N ratio of 16:1 was capable of producing the nitrite concentration below 0.1 mg N/L. Acceptable nitrite concentrations (i.e., NO_2^--N < 0.2 mg N/L) were also observed in treatment 1 (i.e., C:N = 2:1). Without any organic carbon, nitrite increased rapidly after day 10 but still at the level below 1.0 mg N/L. According to Fig. 4.2C, nitrate was found in the range from 1.0 to 1.5 mg N/L and did not seem to be influenced by the extent of organic carbon addition.

Figure 4.3 indicated the change in color of water in glass bottles. For the controls (i.e., only nitrogen) and treatment 1 (i.e., C:N = 2:1), phytoplankton flocculated by the end of the experiment, resulting in a clear separation from water. The reason for this observation was still unknown at this stage. However, the measurement of chlorophyll-a in the controls and treatment 1 revealed a slight increase from about 20 to 180 mg/m^3, suggesting a small phytoplankton growth. The rapid phytoplankton bloom occurred in treatment 2 (i.e., C:N = 16:1) since the water changed from light green to darker shading in less than a week. Measurement of chlorophyll-a indicated a larger increase from 15 to 265 mg/m^3, a significant difference compared to other bottles (Fig. 4.4). Phytoplankton proliferation was also accompanied by a substantial increase of suspended solids from 34 to 250 mg SS/L. Substantial increases in chlorophyll-a and suspended solids may imply the direct

assimilation of inorganic nitrogen into phytoplankton and bacterial cells (Burford and Lorenzen, 2004; Avnimelech 2006). The previous literatures also reported a gradual shift in populations from phytoplankton base towards heterotrophic bacteria when organic carbon compounds (e.g. glucose, molasses and tapioca) were regularly supplied into water (Tacon et al., 2002; Hari et al., 2004; Avnimelech, 2006; Ebeling and Timmons, 2007). Heterotrophic bacteria were able to assimilate inorganic nitrogen into their cells directly when the substrate C:N ratios in water were maintained at greater than 5 (Azim et al., 2008). Microscopic examination of suspended solids (i.e., biofloc) in treatment 2 (i.e., C:N = 16:1) revealed that their morphologies were irregular and contained ranges of microorganisms including phytoplankton, filamentous bacteria, rotifers, protozoa and detritus.

The conclusion obtained from this experiment indicated that pond water was more effective than tap water as it already contained microorganisms (e.g., phytoplankton and bacteria) that can immediately utilize carbon for their growth and establish inorganic nitrogen assimilation. Applying high substrate C:N ratio at 16:1 was still effective in maintaining the low level of TAN and nitrite in comparison to lower substrate C:N ratios.
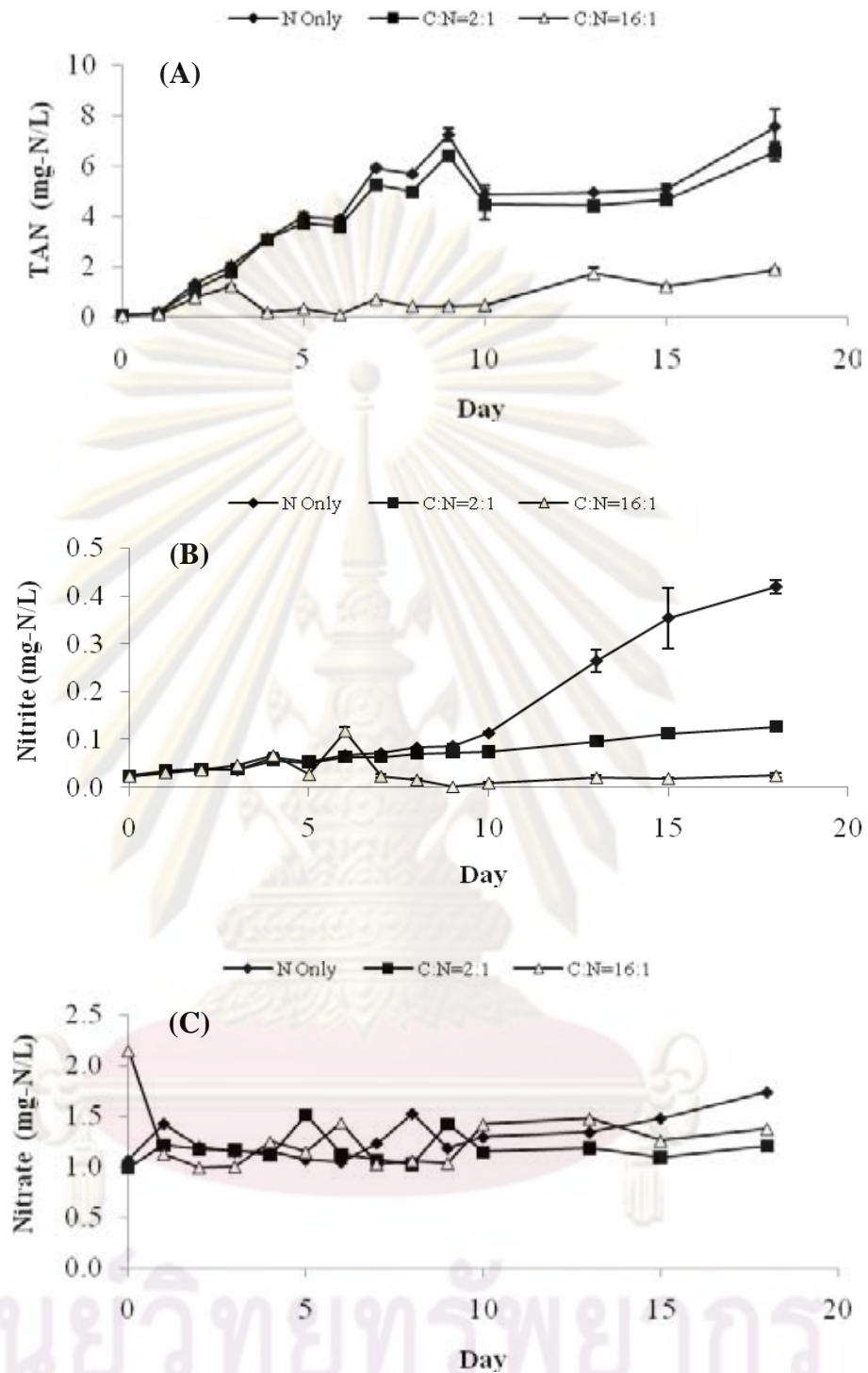
Figure 4.2 The concentration profiles of TAN (A), nitrite (B) and nitrate (C) in experiment 2 that used pond water to provide initial biofloc. Tapioca starch and ammonium chloride were added daily into each glass bottles. (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.
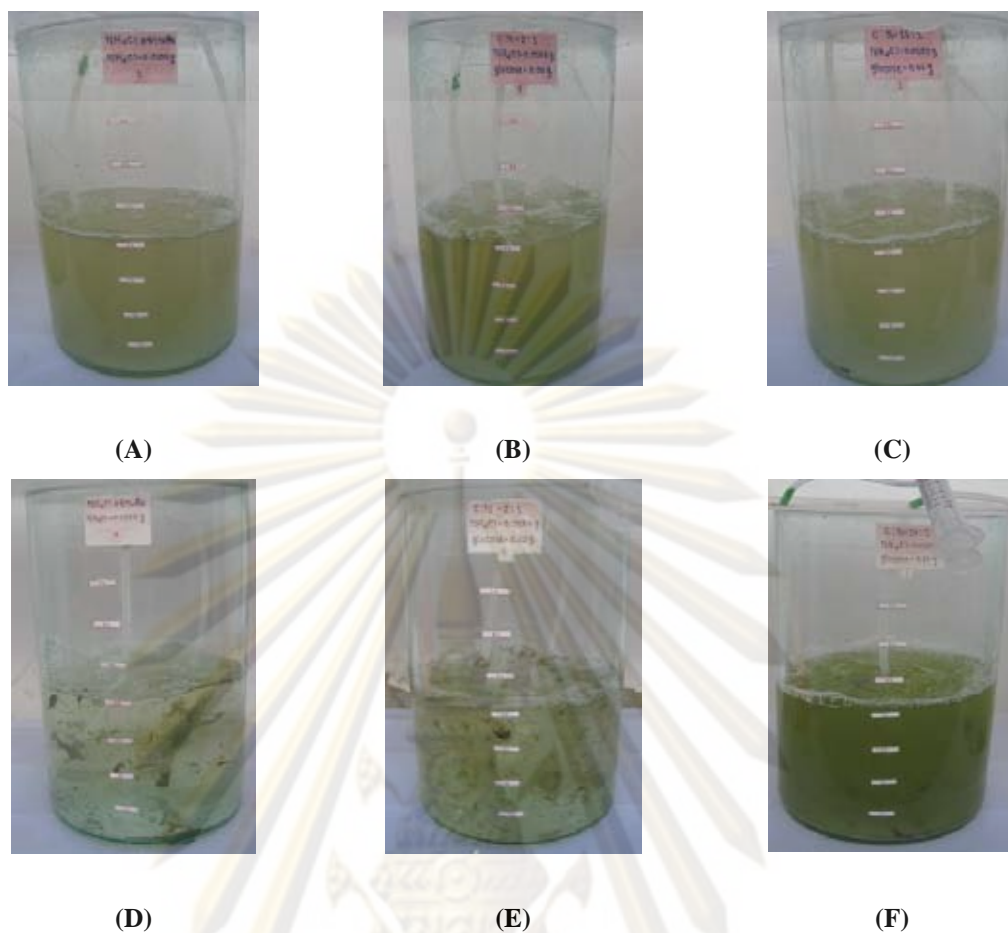
Figure 4.3 Water characteristics after the addition of tapioca starch and ammonium chloride at different C:N ratios: (A) control on day 1 (B) treatment 1 on day 1 (C) treatment 2 on day 1 (D) control on day 18 (E) treatment 1 on day 18 (F) treatment 2 on day 18.
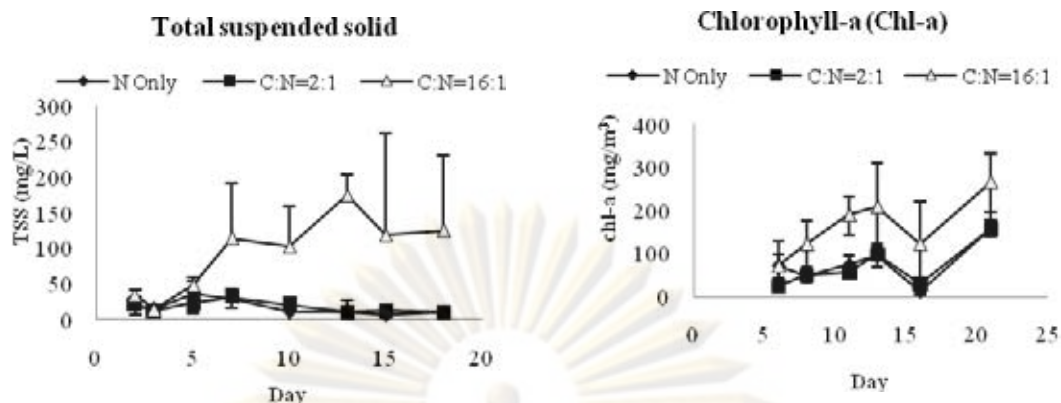
Figure 4.4 Suspended solid concentrations and chlorophyll-a in experiment 2 that used pond water to provide initial biofloc: (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.

4.1.3 Experiment 3: comparison between sources of organic carbon in controlling inorganic nitrogen.

This experiment was carried out to compare the effects of using two different organic carbon compounds, which would be added into the experimental sets daily. Glucose and tapioca starch were chosen for this study. Tapioca starch should be better suit than glucose in the actual practice since it is available in large quantity and cheaper than glucose. Since the purpose of this experiment was only the comparison between glucose and tapioca starch, the tap water was used. Ammonium chloride was used as the nitrogen source and was introduced daily into each bottle at 2.0 mg N/L while carbon was fixed at 16 folds by weight of nitrogen. According to Fig. 4.5(A-C), TAN and nitrite were less than 1.0 mg N/L regardless of organic carbon sources. Nitrate concentrations were observed between 0.8 and 1.6 mg N/L. Detailed examination of the data as shown in Table 4.1 indicated that the average values of TAN were at 0.106 ± 0.177 and 0.034 ± 0.048 mg N/L for treatment 1 (glucose) and treatment 2 (tapioca starch), respectively. The average nitrite concentrations from both systems were similar, measuring at 0.024 ± 0.053 mg N/L for glucose and 0.030 ± 0.043 mg N/L for tapioca starch. The statistical analysis (t-test) performed on the

data indicated that TAN in treatment 1 were insignificantly difference from that in treatment 2 (P > 0.05). Similar result (P > 0.05) was found for the nitrite. Based on the result presented, glucose can be substituted by tapioca starch as the carbon source for microorganisms in water.

Table 4.1 Data for inorganic nitrogen compounds in experiment 3

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Glucose	Starch	Glucose	Starch	Glucose	Starch
1	0.006	0.040	0.015	0.000	0.855	1.157
2	0.070	0.005	0.007	0.071	0.909	0.979
3	0.005	0.148	0.002	0.009	0.756	1.018
4	0.007	0.008	0.010	0.004	0.819	1.220
5	0.091	0.023	0.007	0.041	1.039	1.283
7	0.527	0.029	0.000	0.000	0.691	0.832
9	0.003	0.018	0.000	0.000	0.997	1.191
11	0.141	0.003	0.154	0.116	0.989	0.949
Average	0.106±0.177	0.034±0.048	0.024±0.053	0.030±0.043	0.882±0.124 [a]	1.079±0.157 [a]
T-Test	P=0.1610		P=0.3116		P=0.0028	

[a] Indicated statistically insignificant differences (P < 0.05)
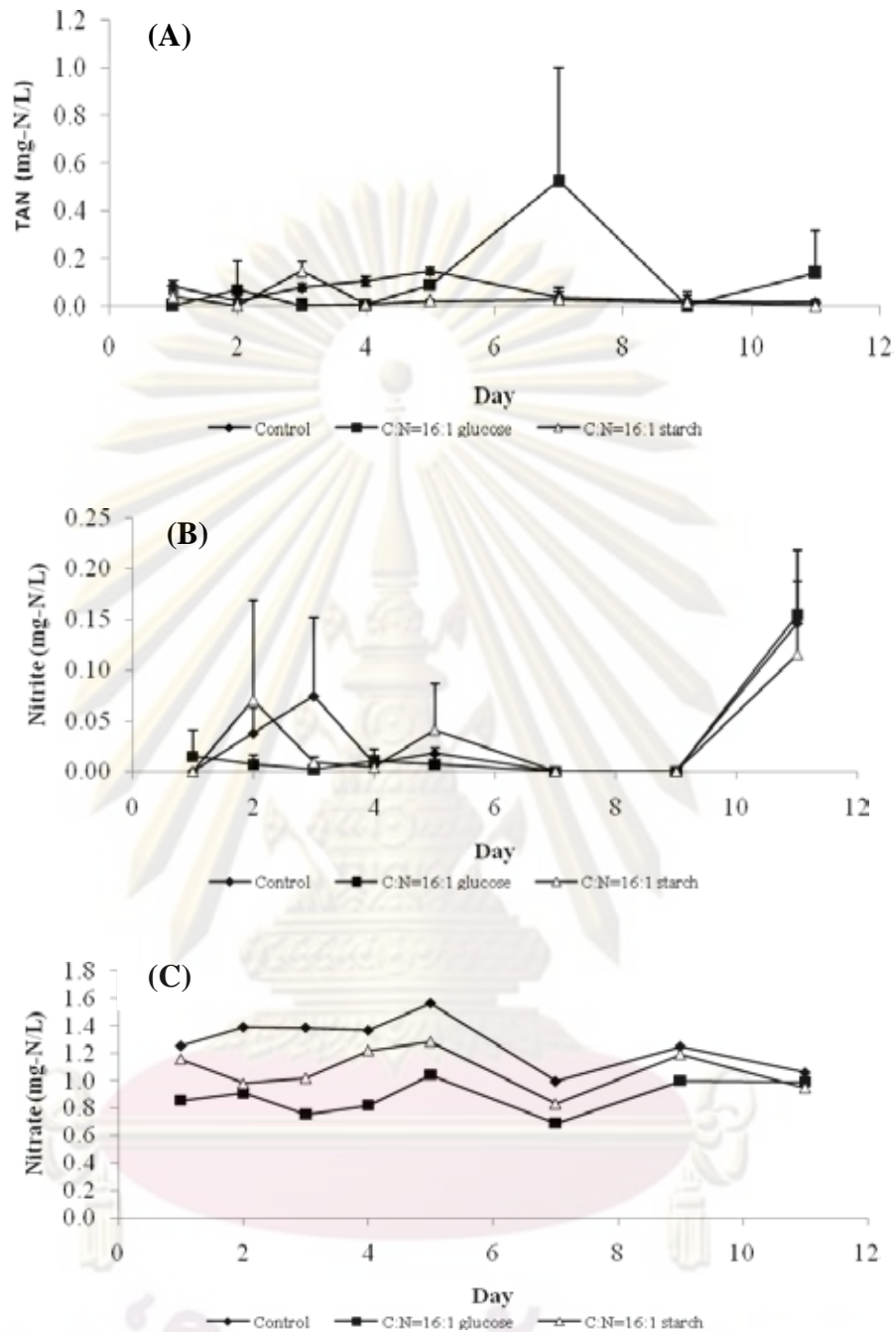
Figure 4.5 A-C Inorganic nitrogen profiles from the bottles that were dially supplied
with glucose or tapioca starch: (Control) daily addition of nitrogen only, (Treatment
1) daily addition of glucose at C:N = 16:1 and (Treatment 2) daily addition of tapioca
starch at C:N = 16:1.

4.1.4 Experiment 4: effect of manipulating the substrate C:N ratio with starch as sole organic carbon source.

In the present experiment, the effect manipulating of substrate C:N ratios was investigated with tapioca starch as the sole organic carbon source. Nitrogen from ammonium chloride (80%) and commercial shrimp diets (20%) were added on the daily basis into each glass bottle to achieve the nitrogen dose at 1.0 mg N/L. The shrimp feed was used as an additional nitrogen source because it contained essential trace elements and metals required for bacterial growth. According to Fig. 4.6A, no significant difference was observed for TAN profiles for each C:N ratio. The maximum TAN concentrations were reached for each treatment on day 5 at approximately 5.0 mg N/L before rapidly decreased below 1.0 mg N/L by day 13. Treatment 2 (i.e., C:N = 16:1) appeared more effective than the controls and treatment 1 in controlling nitrite. Nonetheless, nitrite concentrations remained less than 0.3 mg N/L in all treatments for the entire experiment (Fig. 4.6B). Similarly for nitrate (Fig. 4.6C), no significant difference was observed between the controls and treatments. Nitrate concentrations ranged from 1 to 2 mg N/L.

Better inorganic nitrogen controls in treatment 2 (i.e., C:N = 16:1) compared to experiment 2 could be linked to the greater biofloc production. In this study, the suspended solids in treatment 2 (i.e., C:N = 16:1) increased rapidly from less than 25 to 800 mg SS/L, whereas significantly lower suspended solids were observed in the control and treatment 1 (Fig. 4.7). Chlorophyll also increased during the experiment but it was the chlorophyll-c that accounted for the highest amount at the end of the experiment. By the end of the experiment, the levels of chlorophyll-a, chlorophyll-b, and chlorophyll-c in treatment 2 were measured at 1,047, 812 and 2,233 mg/m^3, respectively. The presence of chlorophyll-c suggested that diatoms developed into the dominant phytoplankton population in water. The effects that influenced the population dynamics of phytoplankton needed to be further investigated in the future. Similar to experiment 2, the increase of suspended solids and chlorophyll was an indication that inorganic nitrogen controls were taken place based on the direct assimilation. Starch promoted the growth of heterotrophic bacteria and phytoplankton and during that process inorganic nitrogen treatment was established. Table 4.2 summarizes water quality data measured during experiment 4.
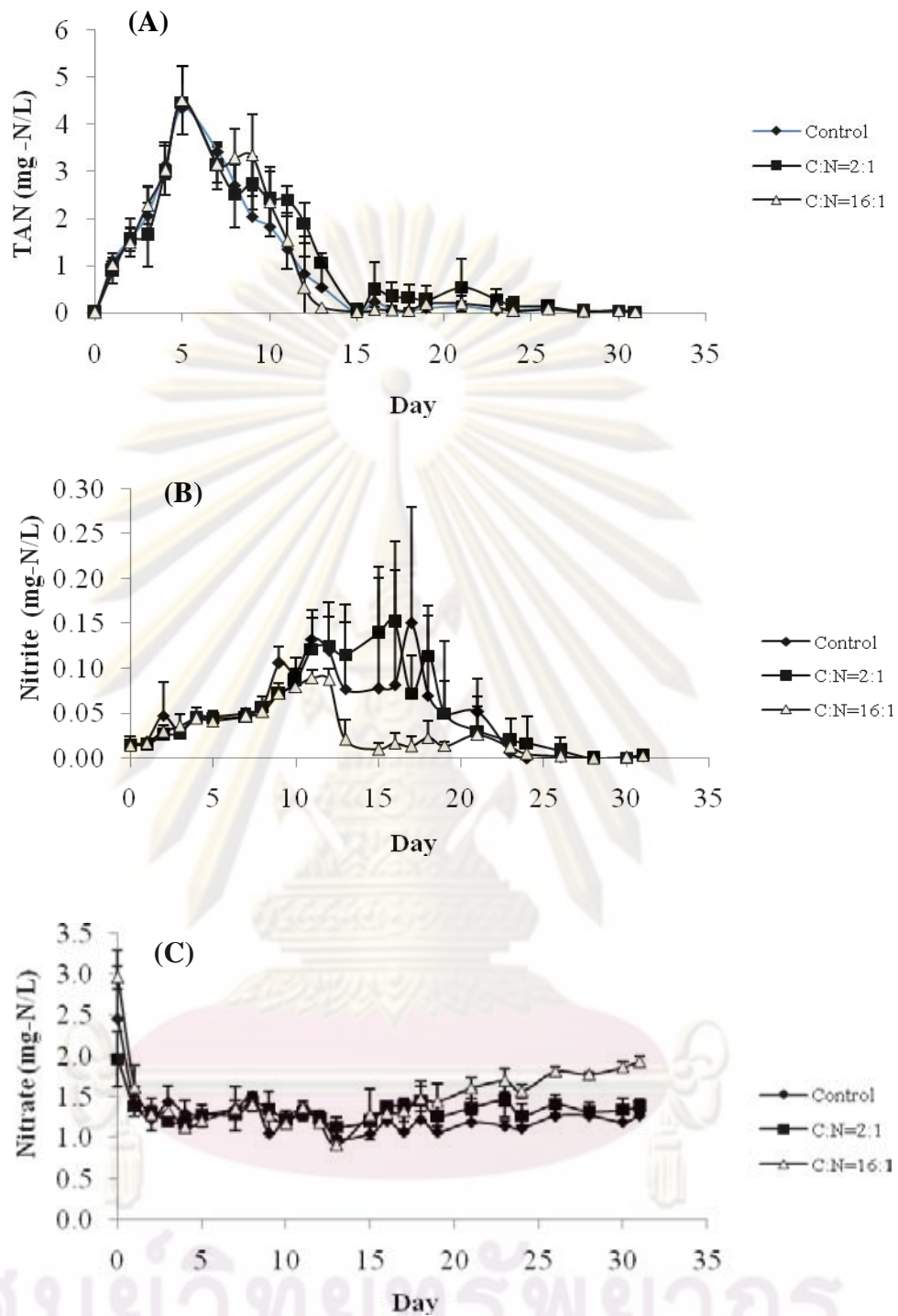
Figure 4.6 A-C Inorganic nitrogen profiles in experiment 4 that used tapioca starch as a sole carbon source and ammonium chloride and shrimp diets as combined nitrogen sources. Substrates were added into glass bottles daily. (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.
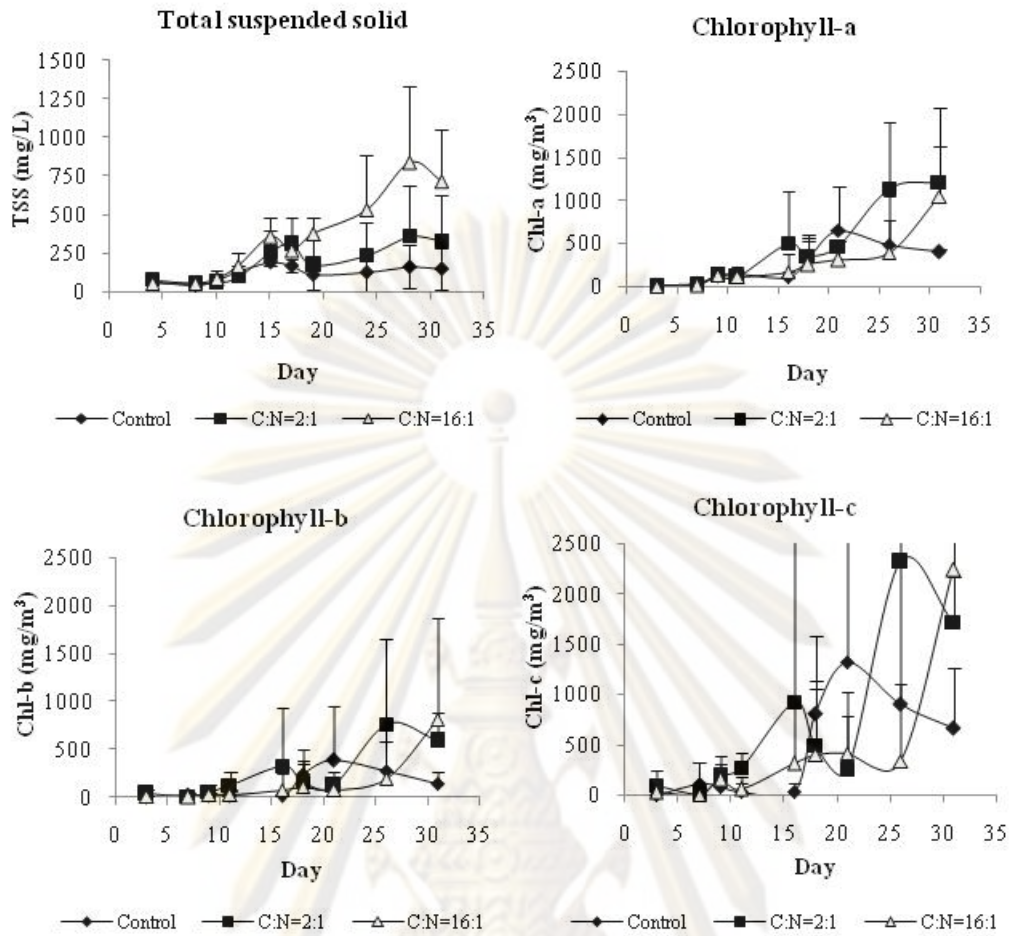
Figure 4.7 Suspended solid concentrations and chlorophyll in experiment 4 that used tapioca starch as a sole carbon sources and ammonium chloride and shrimp diets as combined nitrogen sources. Substrates were added daily. (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.

Table 4.2 The data of water quality parameters measured in the experiment 4. Display is the mean±SD *(min.-max.)* of three replications.

Parameters	Carbon to nitrogen ratio		
	Control (N Only)	Treatment 1 (C:N =2:1)	Treatment 2 (C:N =16:1)
Temp. (^{o}C)	31.90±0.36 *(26.77-37.10)*	32.18±0.34 *(27.00-37.40)*	32.21±0.11 *(26.87-37.40)*
pH	9.00±0.07 *(8.47-9.65)*	8.99±0.08 *(8.40-9.68)*	8.90±0.11 *(8.31-9.57)*
Alkalinity (mg CaCO$_3$/L)	83.33±10.81 *(46.67-126.67)*	85.48±7.68 *(46.67-120.00)*	93.33±8.63 *(63.33-120.00)*
TSS (mg/L)	122.67±51.04 *(42.71-191.57)*	198.06±114.46 *(58.18-360.00)*	345.64±165.37 *(53.38-841.14)*
Chlorophyll-a (mg/m^3)	249.28±215.70 *(0.00-651.15)*	437.30±279.90 *(21.51-1208.52)*	269.20±318.09 *(0.00-1047.03)*
Chlorophyll-b (mg/m^3)	123.58±217.90 *(0.00-384.03)*	241.27±287.73 *(9.31-764.35)*	146.14±332.20 *(0.00-811.71)*
Chlorophyll-c (mg/m^3)	440.28±747.42 *(0.00-1325.64)*	696.64±849.90 *(7.11-2314.33)*	435.82±787.42 *(0.00-2233.12)*

4.2 The zero-water exchange Tilapia cultivation in biofloc technology system using the information from section 4.1.

4.2.1 Experiment 5: effect of organic carbon addition in controlling inorganic nitrogen for Tilapia culture in closed water system.

In this experiment, the zero-water exchanged Tilapia cultivation was performed to assess the feasibility of using the information obtained in section 4.1. The physical parameters of water in the controls were reported as followed; pH = 6.7 – 9.6, temperature = 27.1 – 35.9 ºC and alkalinity = 43 – 157 mg/L $CaCO_3$. The values of these parameters for the treatments were; pH = 7.9 – 8.1, temperature = 32.5 – 32.6 ºC and alkalinity = 99 – 128 mg/L $CaCO_3$. Despite trying to maintain proper operating conditions by a constant aeration and adding $NaHCO_3$ to control pH, it was still unable to keep suitable condition for Tilapia growth as can be seen by the values of pH and alkalinity above 8.5 and 32 ºC for a lengthy period of cultivation. This experimental outcome may imply that biofloc technology systems were susceptible to variation in operating conditions and required controlling mechanisms to keep suitable water quality. Figure 4.8 illustrates the effects of starch addition on inorganic nitrogen profiles. For the controls, TAN increased slowly from negligible levels (i.e., TAN < 0.5 mg N/L) to reach the maximum value at 18.99 mg N/L on day 12. The average TAN concentration in that period was at 16.39 ± 1.16 mg N/L. A rapid nitrite buildup was clearly noticeable after TAN concentration started to decline. Nitrite accumulation lingered at high levels (i.e., NO_2^--N > 5.0 mg N/L) for 26 days with the average nitrite concentration in this period at 76.48 ± 4.68 mg N/L. Substantial nitrate production coincided with the period when nitrite concentration began to diminish. Insignificant levels of TAN and nitrite (i.e., TAN < 0.5 mg N/L and NO_2^--N < 0.5 mg N/L) was accompanied by an increasing nitrate after day 31 until the conclusion of experiment. Similar inorganic nitrogen profiles were observed in treatment tanks. For treatment 1 (i.e., C:N = 2:1), the average TAN during the first 2 week was at 12.97 ± 1.12 mg N/L, which was slightly lower than the controls. The peak TAN appeared on day 12 at 15.43 ± 1.56 mg N/L. Nitrite also accumulated at significant levels between day 14 and 27 at 38.36 ± 4.93 mg N/L with peak measured

at 77.70 ± 6.60 mg N/L on day 26. The continued production of nitrate along with negligible concentrations of TAN and nitrite were observed after day 33 until the conclusion of the experiment. For treatment 2 (i.e., C:N = 16:1), the average TAN during the first 3 week was found at 2.74 ± 1.62 mg N/L, which is significantly lower than the controls. Nitrite also accumulated in treatment 2 at high levels between day 14 and 29 with the average nitrite concentration in this period measured at 25.97 ± 8.71 mg N/L. Nitrate production was apparent after nitrite began to diminish after day 32. The final nitrate concentration in treatment 2 was 33.37 ± 20.20 mg N/L.

The effective control of TAN and nitrite occurred after the complete nitrification was established on day 33. Inorganic nitrogen control in aquaculture ponds occurred based on the following biological processes; (1) photoautotrophic removal microalgae, (2) immobilization by heterotrophic bacteria to produce new biomass proteins and (3) chemoautotrophic oxidation to nitrate by nitrifying bacteria (Azim and Little, 2008). Despite the daily addition of starch at high C:N ratio (i.e., C:N = 16:1) into cultivating tanks, dangerous levels of TAN and nitrite stilled lingered in the treatments for extended periods that may affect fish welfare. The average survival rate in treatment 2 was at 38.70 ± 32.82%. Large deviation could be linked to Tilapia fighting each other causing injuries. Two out of three replications for both the controls and treatment 1 were unable to sustain any Tilapia survival (i.e., survival rate = 0%). Moreover, the average daily growth (ADG) of Tilapia in the controls, treatment 1 and treatment 2 were determined at 0.72 ± 0.63, 0.61 ± 0.53 and 1.23 ± 0.26 g/day, which was significantly lower than other biofloc systems that reported ADG in the range from 3.05 to 3.53 g/day (Little et al., 2008) but in line with the result from Azim et al., 2008. Possible reasons for low growth rate were: (1) the inability of the cultivating system to maintain acceptable TAN and nitrite concentrations during the startup period, (2) the extremely high suspended solids levels that may damage gills and hinder the visibility of Tilapia to obtain food, (3) the relatively small containers used in the experiment that limited Tilapia swimming and (4) the Tilapia fighting and biting with each other. Table 4.4 demonstrates the Tilapia growth data for experiment 5.

According the described result, inorganic nitrogen profiles displayed the sequential buildups of TAN followed by nitrite, a characteristic usually encountered

during the startup of nitrifying systems. Negligible TAN and nitrite concentrations and the steady increase of nitrate signaled the establishment of a complete nitrification. The daily supplement of starch into treatment 2 appeared to increase the suspended solids levels at the greater extent than the controls and treatment 1 (Fig. 4.10). The maximum suspended solids for the controls, treatment 1 and treatment 2 were found on day 30 at 636, 708 and 907 mg SS/L, respectively. For all experiments, the suspended solid concentrations increased with respect to increasing quantity of starch up to day 30 before starting to move up and down. This fluctuation was perhaps due to the gravitational sedimentation and resuspension by fish swimming. According to Fig. 4.9, the color of water on day 4 for all conditions was orange due to a reflection from the bottom of container. The color of water for all experimental sets changed from brown to dark green during the first week as a result of phytoplankton bloom. The chlorophyll contents reached the maximum levels around 2,000 to 2,600 mg/m^3 on day 10 for all bottles before decreased to steady values after the third week (Fig. 4.10). The decrease in chlorophyll contents after day 10 corresponded to the change in water color from dark green to lighter shading especially in treatment 2 as shown in Figure 4.9. The fluorescent microcopy of biofloc samples also revealed that lesser amount of chlorophyll (red fluorescence) was presence in biofloc as more starch was added into water particularly in treatment 2 (Fig. 4.11). Decreasing chlorophyll in treatment 2 suggested the population shifts from phytoplankton-based systems towards nitrifying or heterotrophic bacterial systems. This observation concurred with the result by Boyd and Clay (2002) and Tacon et al. (2002).
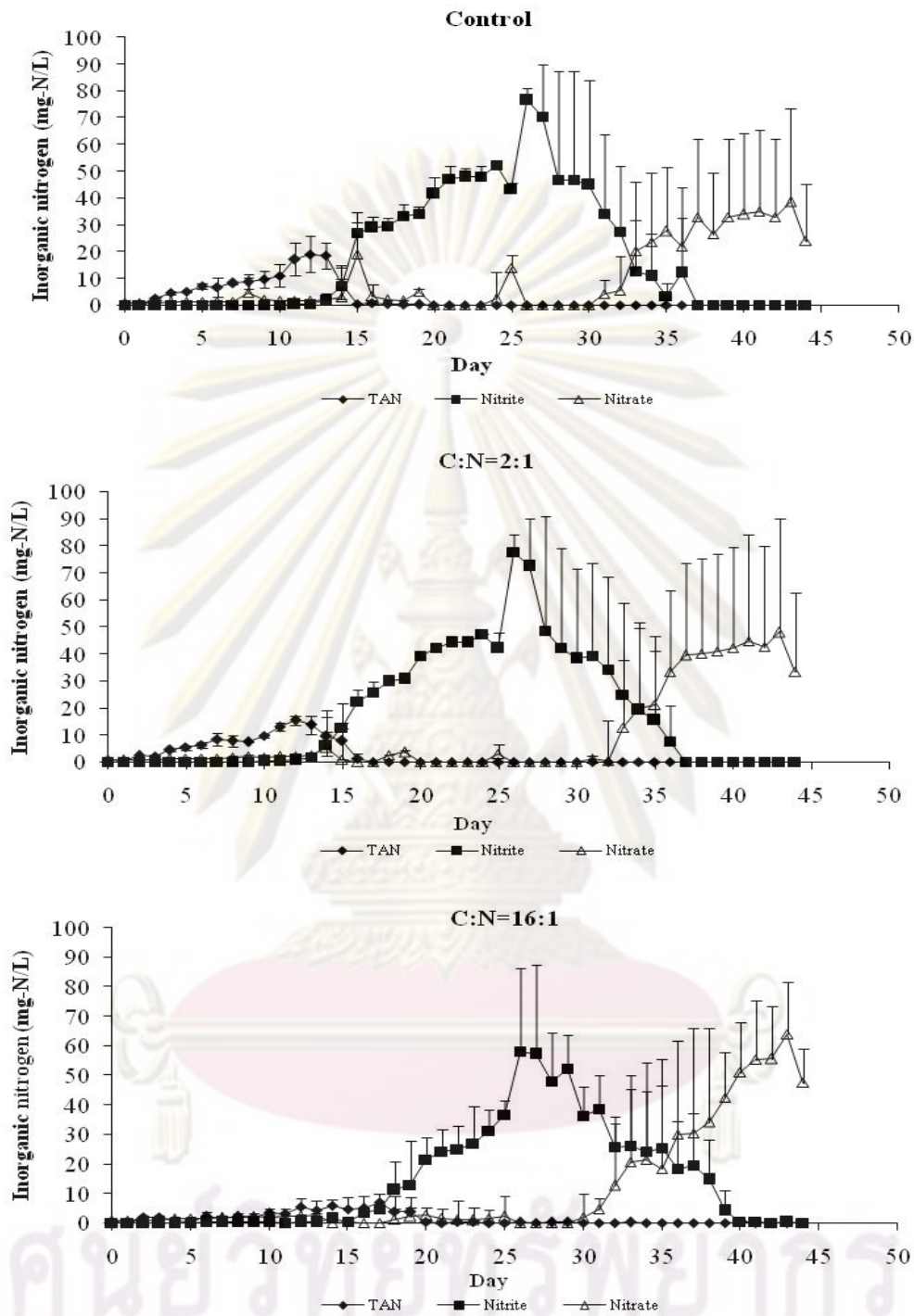
Figure 4.8 Inorganic nitrogen profiles during the zero-water exchanged Tilapia cultivation in 130 L containers: (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1
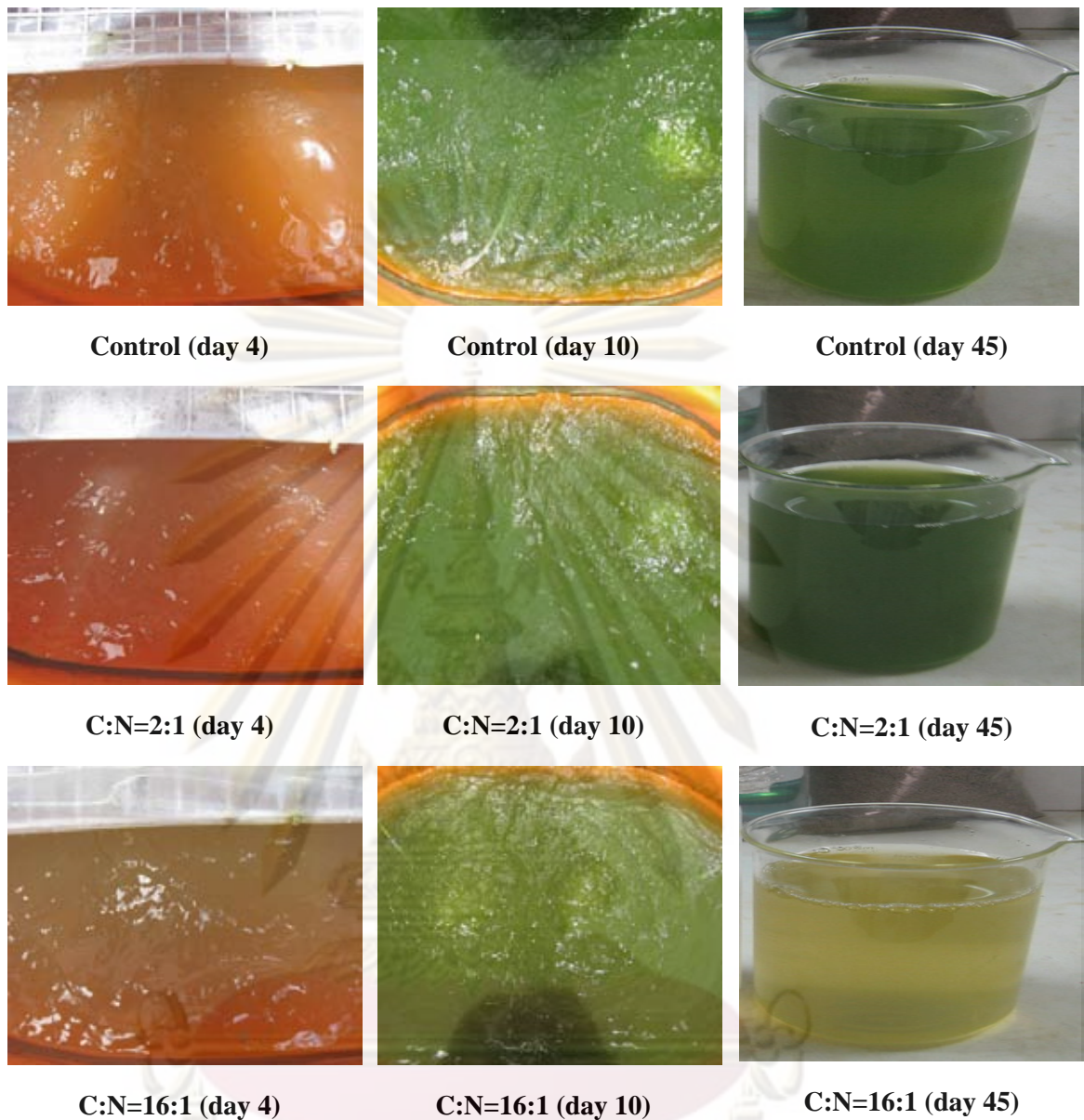
Figure 4.9 Water characteristics after the addition of substrate at different C:N ratio:
(upper row) controls on day 4, 10 and 45 (middle row) Treatment 1 on day 4, 10 and
45 and (bottom row) Treatment 2 on day 4, 10 and 45.
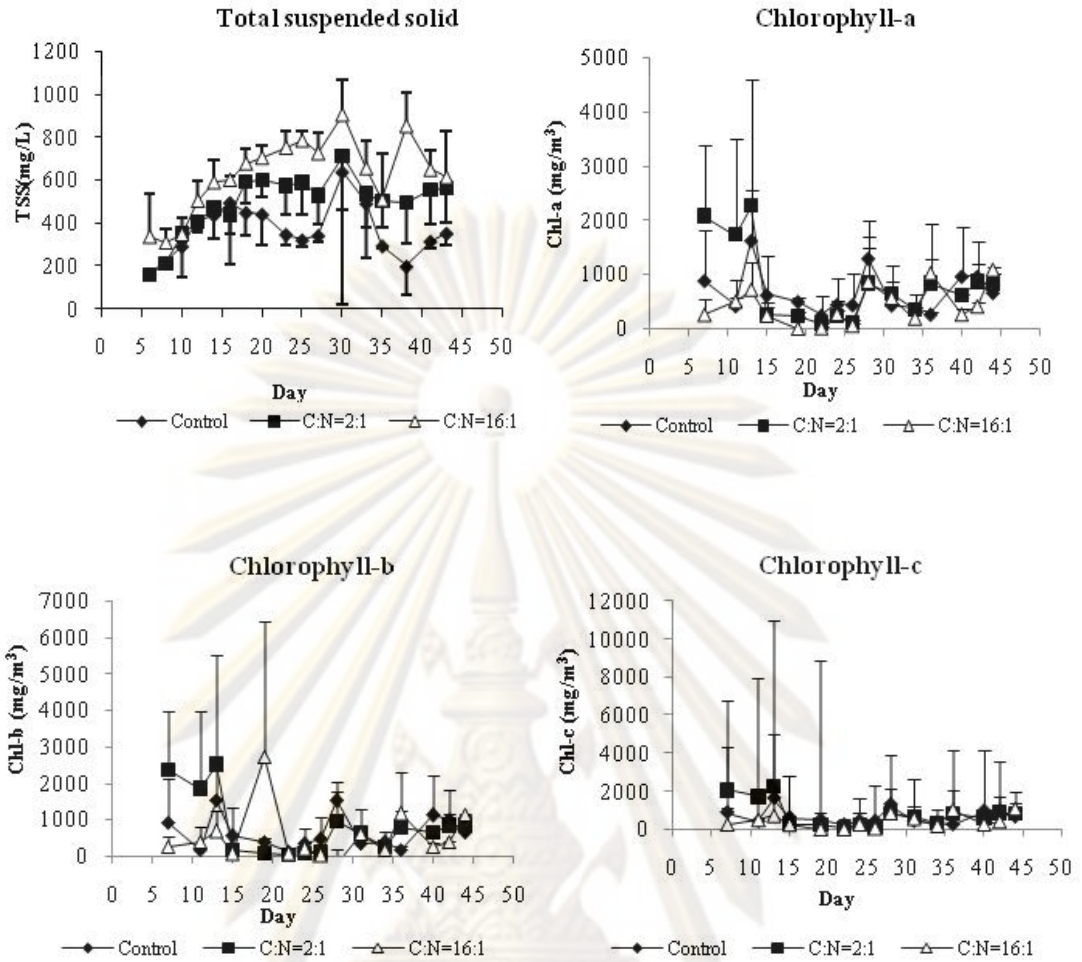
Figure 4.10 Suspended solid and chlorophyll concentration in water during the zero-water exchange Tilapia cultivation in 130 L containers (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.

Control (day 37)

Control (day 37)

C:N=2:1 (day 37)

C:N=2:1 (day 37)

C:N=16:1 (day 37)

C:N=16:1 (day 37)
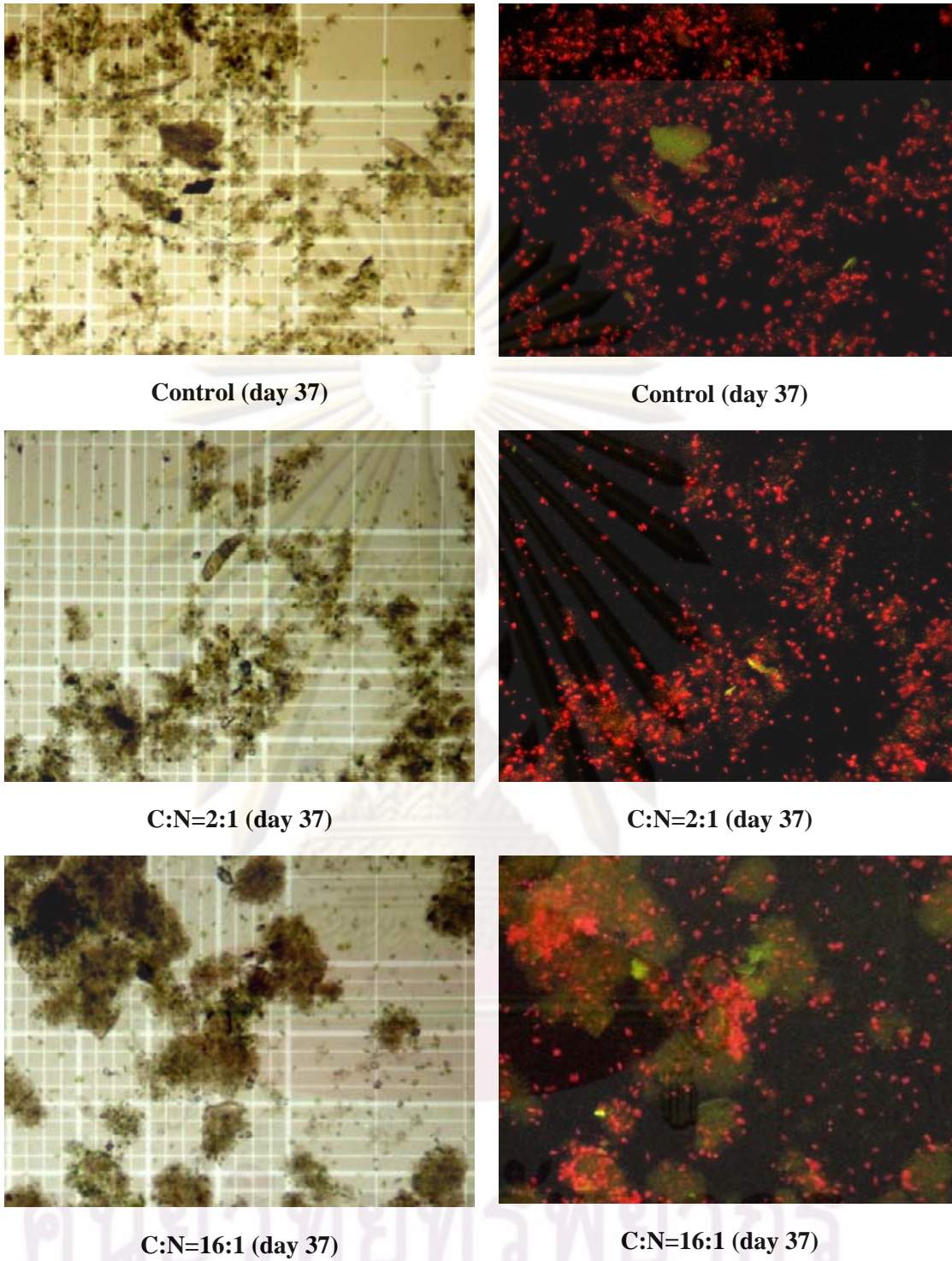
Figure 4.11 The Fluorescent microcopy of biofloc samples taken on day 37 from the controls (nitrogen only), treatment 1 (C:N = 2:1) and treatment 2 (C:N = 16:1).

Table 4.3 Water characteristics during the zero-water exchange Tilapia cultivation in 130 L containers.

Parameters	Average±SD *(min.-max.)*		
	Control (N-only)	Treatment 1 (C:N=2:1)	Treatment 2 (C:N=16:1)
DO (mg O_2/L)	5.97±0.19 *(4.91-7.07)*	5.97±0.07 *(4.99-7.02)*	5.82±0.15 *(4.66-7.03)*
Temperature (°C)	31.41±0.31 *(27.05-35.90)*	32.58±0.30 *(27.30-35.60)*	32.45±0.23 *(27.37-35.53)*
pH	8.12±0.15 *(6.67-9.64)*	8.00±0.17 *(6.50-8.74)*	7.99±0.17 *(6.00-8.53)*
Alkalinity (mg $CaCO_3$/L)	97.67±15.25 *(43.33-156.67)*	99.17±11.20 *(46.67-150.00)*	127.62±23.47 *(43.33-203.33)*
Floc Volume (mL)	18.50±3.71 *(8.00-33.00)*	29.28±11.00 *(1.70-43.50)*	55.50±11.85 *(18.00-82.33)*
TSS (mg/L)	354.42±148.81 *(163.83-636.04)*	497.46±65.24 *(159.68-908.01)*	619.98±60.19 *(310.00-906.88)*
Chlorophyll-a (mg/m^3)	674.00±333.00 *(241.00-1626.00)*	793.47±727.23 *(108.87-2253.94)*	430.55±333.64 *(0.00-1089.14)*
Chlorophyll-b (mg/m^3)	643.00±408.00 *(112.00-1546.00)*	794.56±930.64 *(24.53-2490.50)*	521.53±939.52 *(18.52-2712.32)*
Chlorophyll-c (mg/m^3)	1689.00±1210.00 *(196.00-4232.00)*	2144.12±2690.55 *(3.925-6883.92)*	1377.23±2286.81 *(10.56.6453.81)*
TAN (mg-N/L)	2.87±1.88 *(0.00-18.99)*	2.60±1.53 *(0.00-15.43)*	1.41±1.43 *(0.00-6.98)*
Nitrite (mg-N/L)	18.41±11.59 *(0.001-76.48)*	18.02±13.09 *(0.002-77.70)*	14.48±8.28 *(0.002-58.13)*
Nitrate (mg-N/L)	9.58±11.89 *(0.00-38.58)*	10.17±14.84 *(0.00-48.02)*	11.72±11.60 *(0.00-63.90)*

Table 4.4 Tilapia growth data during the zero-water exchanged Tilapia cultivation in 130 L fiber-glass containers.

Parameters	Average±SD *(min-max)*		
	Control (N-only)	Treatment 1 (C:N=2:1)	Treatment 2 (C:N=16:1)
Average initial weight (g)	45.69±2.66 *(43.89-48.75)*	44.06±4.89 *(39.00-48.75)*	44.73±3.88 *(41.00-48.75)*
Average initial length (cm)	13.44±0.28 *(13.19-13.74)*	13.37±0.77 *(12.5-13.94)*	13.42±0.48 *(12.87-13.76)*
Initial density (kg/m^3)	3.04±0.04 *(3.00-3.08)*	3.03±0.04 *(3.00-3.08)*	3.08±0.08 *(3.00-3.16)*
Average final weight (g)	63.33±55.08 *(0.00-100.00)*	55.33±39.20 *(0.00-86.00)*	100.00±14.53 *(83.33-110.00)*
Average final length (cm)	11.17±9.67 *(0.00-17.00)*	10.70±9.28 *(0.00-16.42)*	17.39±1.98 *(15.57-19.95)*
Final density (kg/m^3)	0.73±0.42 *(0.00-0.77)*	1.31±1.90 *(0.00-3.30)*	2.56±2.11 *(0.85-4.92)*
Survival rate (%)	7.87±6.85 *(0.00-12.50)*	21.85±29.61 *(0.00-55.56)*	38.70±32.82 *(11.11-75.00)*
ADG (g/day)	0.72±0.63 *(0.00-1.14)*	0.61±0.53 *(0.00-0.92)*	1.23±0.26 *(0.94-1.46)*

4.2.2 Experiment 6: zero-water exchange Tilapia cultivation in biofloc systems – an improvement from experiment 5

The physical parameters for production water in the controls were reported as followed: pH = 8.83 ± 0.47, temperature = 29.07 ± 0.19 ºC and alkalinity = 115.67 ± 5.26 mg/L $CaCO_3$. The values of these parameters for the treatments were similar and they were pH = 8.51 ± 0.04, temperature = 29.32 ± 0.10 ºC and alkalinity = 137.33 ±9.55 mg/L $CaCO_3$. The average value of pH for both the treatments fell outside the optimal range for Tilapia growth. Hence, it is necessary to have the effective control for this particular parameter during the intensive aquacultures in biofloc technology

systems. Figure 4.12 illustrates the effects of tapioca starch addition on inorganic nitrogen profiles. For the controls, TAN increased slowly from negligible levels (i.e., TAN < 0.5 mg N/L) to reach the maximum value at 26.16 mg N/L on day 14. The average TAN concentration in that period was at 11.79 ± 8.95 mg N/L. A rapid nitrite buildup was clearly noticeable after TAN started to decline. Nitrite accumulation in the controls lingered at high levels (i.e., $NO_2^--N > 5.0$ mg N/L) for 26 days with the average nitrite concentration in this period at 29.0 ± 12.24 mg N/L. Substantial nitrate production coincided with the period when nitrite concentration began to diminish. Insignificant levels of TAN and nitrite (i.e., TAN < 0.5 mg N/L and $NO_2^--N < 0.5$ mg N/L) was accompanied by an increasing nitrate after day 47 until the conclusion of experiment. Similar inorganic nitrogen profiles were observed in the treatments. In this case, the average TAN concentration during the first 2 week was at 3.6 ± 1.63 mg N/L while the peak TAN appeared on day 24 at 16.92 mg N/L. Nitrite also accumulated at significant levels between day 26 and 45 at 25.74 ± 16.11 mg N/L with the peak value measured at 51.05 mg N/L on day 39. Nitrate production accompanied by negligible TAN and nitrite (i.e., TAN < 0.5 mg N/L and $NO_2^--N < 0.5$ mg N/L) were observable after day 47. According to the experimental result described, the sequential buildups of TAN followed by nitrite were common during the startup of nitrifying systems. These inorganic nitrogen accumulations were the result of unequal growth rates between ammonia and nitrite oxidizing bacteria. Negligible buildup of TAN and nitrite accompanied by an increasing trend of nitrate was a good indication for a complete nitrification. The result described indicated that it took approximately 7 weeks for a complete nitrification to proceed in this work. This duration concurred with other experiments, which reported 5 to 8 weeks to establish nitrification in suspended-growth aquaculture systems (Thakur and Lin, 2003; Hari et al., 2006).

The removal of inorganic nitrogen in biofloc technology systems was conceptualized based on the direct assimilation of inorganic nitrogen during heterotrophic bacterial growth, which required a regular supply of organic carbon compound at high C:N ratios (Avnimelech, 2006). Since starch addition (i.e., C:N = 16:1) was carried out on the daily basis in this work, it was expected that the proliferation of heterotrophic bacteria would be observed along with low TAN and

nitrite concentrations. According to Fig. 4.13, the daily supplement of starch into the treatments appeared to affect the extent of biofloc formation. Biofloc (measured as suspended solids) in the treatments increased from 52 to 1,181 mg SS/L whereas it remained at 99 ± 17 mg SS/L since day 22 for the controls. Biofloc volumes were negligible on the first day of experiment. Final biofloc volumes, measured on day 60, were at 16 and 86 mL/L for the controls and treatments, respectively. In spite of substantial suspended solid formation (Fig. 4.13), the average TAN and nitrite in treatment systems remained at dangerous levels for a lengthy period during the system startup (i.e., day 1 to 45) to suggest that the daily tapioca starch addition to induce inorganic nitrogen assimilation was not fully effective. Almost 89% of Tilapia in the controls was found death while slightly lower death rate at 10% was associated with the treatments. The prolonged exposure to excessive TAN and nitrite was the primary suspects for the death of Tilapia. Successful TAN and nitrite controls occurred after day 47 as a result of complete nitrification. Although various heterotrophic microorganisms and fungi were able to perform nitrification but the rates were slow to exclude their significances (Verstrate and Alexander, 1973; Watson et al., 1981). Based on the experimental outcome, it appeared that nitrification was able to exert a greater role than the direct assimilation by heterotrophic microorganisms. This result disagreed with the conclusions from earlier works that pointed to the importance of heterotrophic bacteria and encouraged their activity in biofloc ponds (Avnimelech, 2006; Azim et al., 2008). It was possible that the biofloc served as the substrate biofilters for nitrifying bacteria to attach. Further study to identify microorganisms in biofloc and their ecological relationships should provide a concise explanation for the experimental result observed. Table 4.5 summaries the values of water parameters measured in this experiment.

A microscopic examination revealed that biofloc samples from the controls and treatments were quite similar. Morphology of biofloc appeared as irregular shape aggregates containing many organisms including filamentous bacteria, protozoa, rotifers, nematodes, small amount of phytoplankton and the remains of detritus (Fig 4.14). The result of PCR-DGGE analysis (Fig. 4.15) demonstrated different emerging patterns for the controls and treatments. These patterns were indicators of dominant bacterial species but they did not provide the total numbers of species available in the

systems. Biofloc samples from the controls and treatments were clearly dominated by different bacterial species for a given sampling date. For a given system, a comparison of band patterns between sampling dates (i.e., day 24, 31, 39, 46, 53 and 60) indicated that dominant bacterial species residing in biofloc evolved with time for both the controls and treatments. Some bands were detectable throughout the study was the indicator that some bacterial species were able to maintain their dominance despite a continuous changing in physical conditions (e.g., pH, DO, temperature, and alkalinity) as well as increasing amounts of substrate added. In the same token, disappearing bands suggested that certain bacteria were unable to flourish under such conditions kept during the Tilapia cultivation. In addition, the diversity of bacterial population in the controls as the cultivation progressed, whereas the opposite was observed in the treatments. The proximate analysis (Table 4.7) demonstrated that the elementary composition of biofloc in a given systems was unchanged with increasing quantity of tapioca starch added. Contents of carbon, hydrogen and nitrogen in biofloc from treatment systems were at 34.5, 4.69 and 4.24% dried weight, respectively while those from control systems were at 21.7, 4.02 and 2.19% dried weight.

The simple nitrogen balance was performed for the controls and treatments after the conclusion of experiment on day 60 (Table 4.8). The cumulative nitrogen input to each Tilapia tank was entirely from feeds while the nitrogen output can be classified as (1) the nitrogen in fish (2) the nitrogen in suspended solids (i.e., biofloc) (3) nitrogen in the form of total dissolved inorganic nitrogen and (4) nitrogen in the form of volatile gases. For the treatments, the cumulative nitrogen input on day 60 was found at 286.07 g N. Of that amount, 54.66 g N (19.11%) was in the form of dissolved inorganic nitrogen (i.e., NH_4^+-N, NO_2^--N, NO_3^--N) with nitrate as the majority (i.e., > 99%). Nitrogen contents in biofloc and Tilapia were at 26.73 g N (9.34%) and 85.29 g N (29.82%), respectively. For the controls, cumulative nitrogen input measured on the last day of experiment was at 76.19 g N. Smaller nitrogen input into the control was due to less feeding as a result of Tilapia mortality on day 45 after the aeration equipment malfunction occurred for an overnight. Similar to treatment systems, nitrate accounted for more than 99% of the dissolved inorganic organic nitrogen (i.e., 33.21 g N) in water. Nitrogen contents of biofloc and fish

biomass in the controls were at 3.02 g N (3.96%) and 34.26 g N (44.97%), respectively. An additional observation from the nitrogen balance was the large nitrogen deficit especially for the treatments. Unaccountable portions of nitrogen were determined at 5.70 g N (7.48%) and 119.39 g N (41.74%) for the controls and treatments. Large values of nitrogen losses as high as 36% were reported for the intensive shrimp cultivation in concrete tanks (Thakur and Lin, 2003). Denitrification and ammonia striping were assumed to be parts of unaccountable portions of nitrogen budget. The occurrence of denitrification implied that oxygen mass transfer limitation may exist at the inner region of biofloc. Lacks of oxygen allowed the possibility for other denitrifying microorganisms such as Anammox to be presence. Finally, the result of nitrogen balance, showing nitrogen contents in biofloc and nitrate at 9.34% and 19.11% of total nitrogen inputs, should reinforce an earlier remark about the importance of nitrification as the principal pathway to remove hazardous nitrogen.

In term of system management aspects, the result obtained implied that water in aquaculture tank must be pre-acclimated to achieve the complete nitrification before being used to grow aquatic animals. With fully acclimated production water, ammonium and nitrite would be converted into nitrate almost immediately, thereby avoiding the excessive buildups of inorganic nitrogen wastes in production systems. De Schryver et al (2008) suggested the range of suspended solids for biofloc technology systems from 200 to 1,000 mg SS/L yet the optimum biofloc level in relation to particular aquaculture species still required further research (Azim and Little, 2008). In addition, supplying organic carbon compounds may be employed as a quick solution to reduce excessive ammonium and nitrite concentrations in ponds. Avnimelech (1999) recommended adding organic carbon compounds into biofloc ponds at the C:N ratio of 20:1. In this experiment, the electrical failure occurred overnight on day 51 causing a depletion of dissolved oxygen that consequently contributed to significant Tilapia death in all cultivating tanks. The result from an independent experiment (Fig 4.16) revealed that the suspended solids from the treatments utilized oxygen in water at 8.30 mg O_2/L/hr resulting in a rapid decrease of DO concentration to a critical level (DO < 0.5 mg O_2/L) within an hour. In contrast, suspended solids from the controls displayed a 10-folds lower oxygen consumption rate at 0.93 mg O_2/L/hr. Therefore, the availability of oxygen in biofloc systems was

critical to the success of aquaculture production and the ability to the system to maintain good water quality. Without sufficient oxygen in water, the input BOD from organic carbon addition cannot be completely stabilized into inert compounds and nitrifying bacteria were unable to carry out the complete nitrification that might lead to excessive accumulation of TAN and nitrite in water.
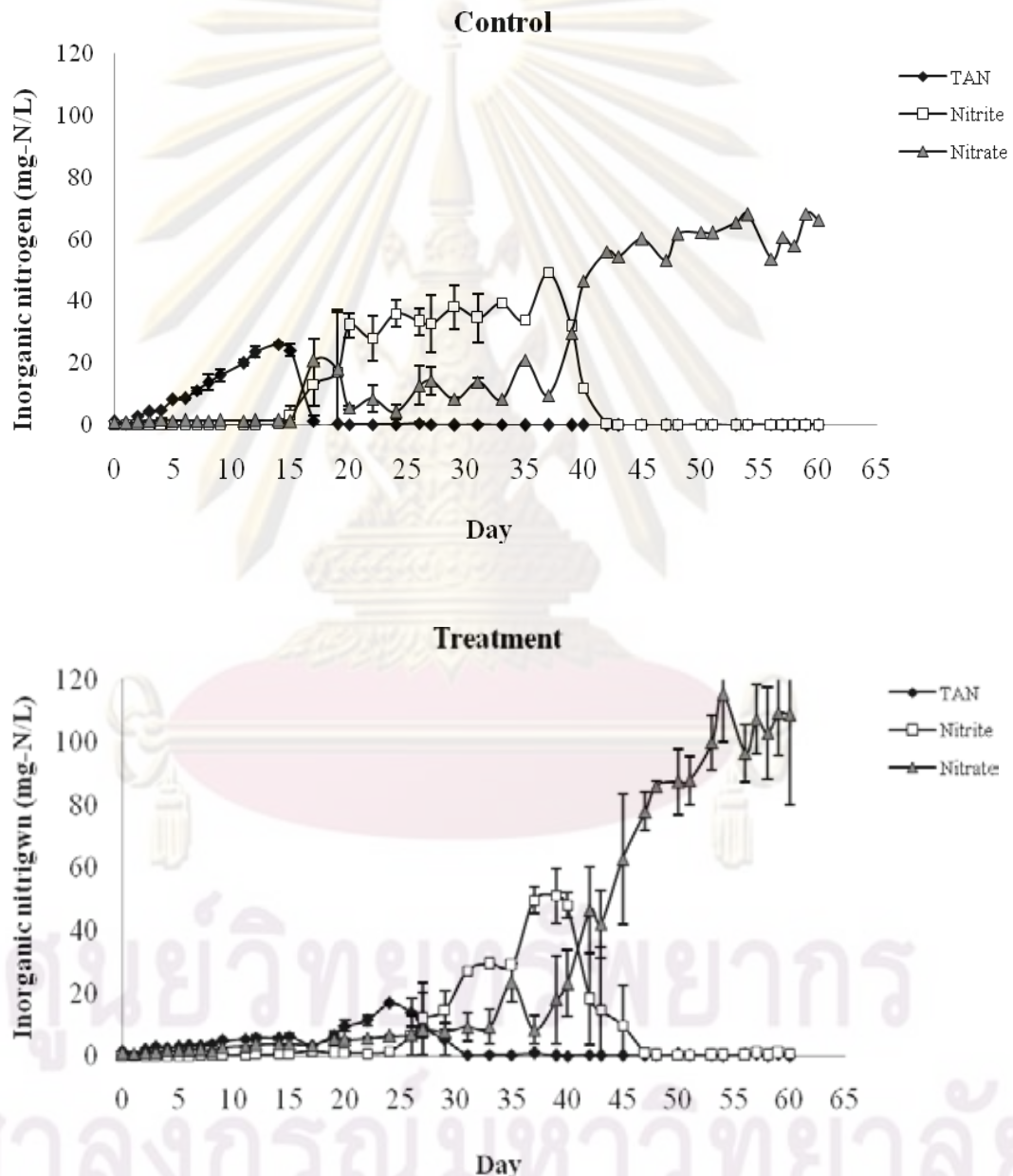
Figure 4.12 Inorganic nitrogen profiles during the zero-water exchanged Tilapia cultivation in 500 L fiber-glass tanks: (Control) feed only and (Treatment) tapioca starch provided daily at C:N = 16:1. Electricity failure occurred on day 51.

Total suspended solid
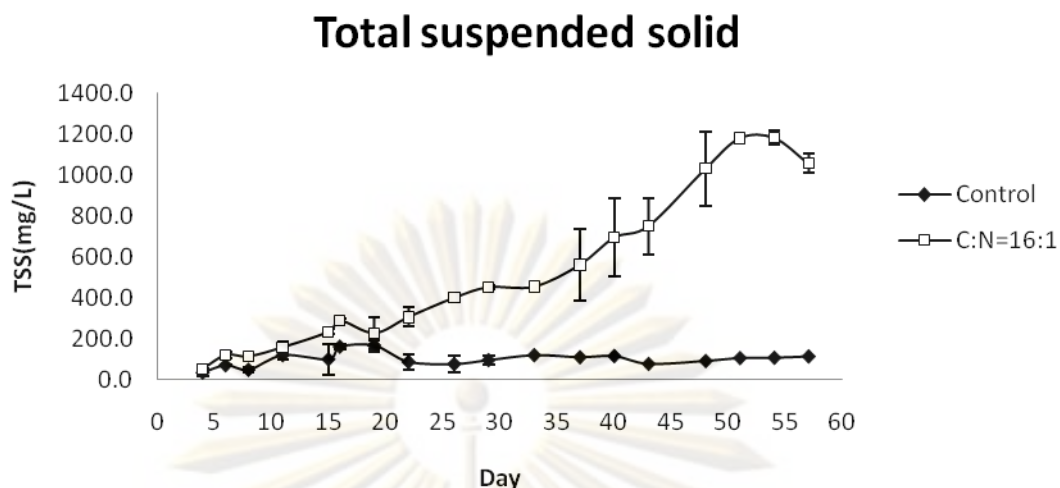
Figure 4.13 Suspended solid concentrations during the zero-water exchanged Tilapia cultivation in 500 L fiber-glass tank: (Control) feed only and (Treatment) tapioca starch provided daily at C:N ratio = 16:1.

ศูนย์วิทยทรัพยากร

จุฬาลงกรณ์มหาวิทยาลัย

Table 4.5 Values of water parameters during the zero-water exchanged Tilapia cultivation in 500 L tanks.

Parameters	Average ± SD *(min.-max.)*	
	Control (N-only)	Treatment (C:N = 16:1)
Temperature (^{o}C)	29.07±0.19	29.32±0.10
	(25.50-33.05)	*(24.75-33.40)*
pH	8.83±0.07	8.51±0.04
	(8.42-9.53)	*(8.38-9.06)*
Alkalinity	115.67±5.26	137.33±9.55
(mg CaCO$_3$/L)	*(55.00-185.00)*	*(90.00-215.00)*
Floc Volume	9.42±2.60	49.29±16.92
(mL)	*(0.30-22.00)*	*(1.35-112.50)*
TSS (mg/L)	107.98±2.22	553.03±69.07
	(34.81-275.68)	*(51.68-1260.62)*
Chlorophyll-a (mg/m^3)	313.15±62.08	769.83±565.31
	(49.03-1915.36)	*(33.06-2916.09)*
Chlorophyll-b (mg/m^3)	413.54±111.01	1277.31±662.94
	(41.90-2317.25)	*(86.51-3524.54)*
Chlorophyll-c (mg/m^3)	1091.10±227.21	2947.57±1254.29
	(23.56-6286.59)	*(17.68-2226.90)*
TAN (mg-N/L)	4.01±0.66	3.14±1.94
	(0.008-26.16)	*(0.01-16.92)*
Nitrite (mg-N/L)	10.39±3.84	7.67±4.65
	(0.005-49.23)	*(0.004-51.05)*
Nitrate (mg-N/L)	25.95±3.32	33.31±6.74
	(0.73-68.27)	*(0.76-109.22)*

Table 4.6 Tilapia growth data from zero-water exchanged Tilapia cultivation in 500 L fiber-glass tanks.

Parameters	Average ± SD *(min-max)*	
	Control (N-only)	Treatment (C:N = 16:1)
Average initial weight (g)	32.20±1.00	31.39±0.78
	(31.49-32.90)	*(30.83-31.94)*
Average initial length (cm)	12.06±0.07	12.09±0.12
	(12.01-12.11)	*(12.00-12.17)*
Initial density (kg/m^3)	3.00±0.05	3.05±0.04
	(2.96-3.03)	*(3.02-3.07)*
Day 50		
(before electricity failure)		
Final density (kg/m^3)	0.78±1.10[*]	10.56±0.87
	(0.00-1.56)	*(9.94-11.17)*
Survival rate (%)	10.87±15.37[*]	95.84±5.89
	(0.00-21.74)	*(91.67-100.00)*
ADG (g/day)	0.45±0.63[*]	1.61±0.03
	(0.00-0.90)	*(1.59-1.63)*
FCR	-[*]	1.00±0.09
		(0.93-1.07)
Day 60		
Average final weight (g)	63.07±89.19[*]	123.58±3.83
	(0.00-126.13)	*(120.87-126.28)*
Average final length (cm)	9.21±13.02[*]	18.62±0.19
	(0.00-18.41)	*(18.48-18.75)*
Final density (kg/m^3)	1.01±1.43[*]	9.59±1.44
	(0.00-2.02)	*(8.57-10.61)*
Survival rate (%)	8.70±12.30[*]	79.32±12.00
	(0.00-17.39)	*(70.83-87.80)*
ADG (g/day)	0.79±1.12[*]	1.54±0.05
	(0.00-1.58)	*(1.50-1.57)*
FCR	-[*]	1.40±0.24
		(1.24-1.57)

(*) Tilapia in control 2 had total mortality on day 31

Floc volume on day 19
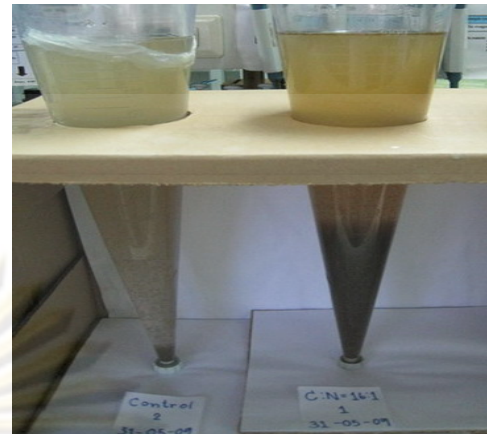
Floc volume on day 60
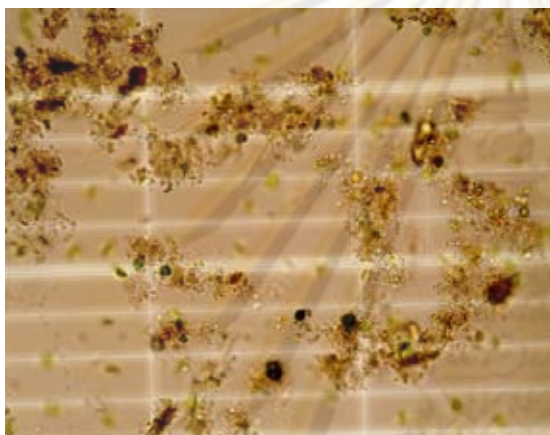
Control (on day 60)
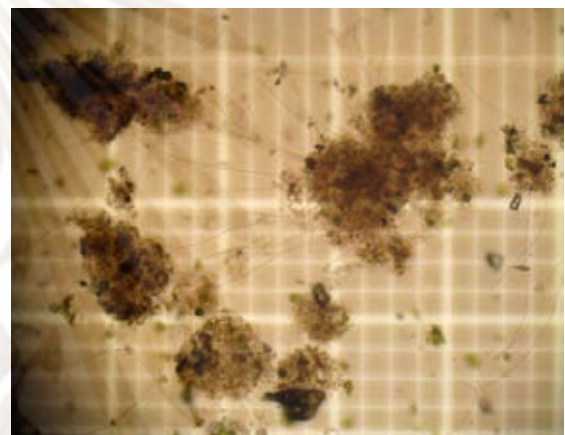
Treatment (on day 60)

Figure 4.14 Biofloc volume and microscopic pictures of biofloc from the controls and treatments on day 60.
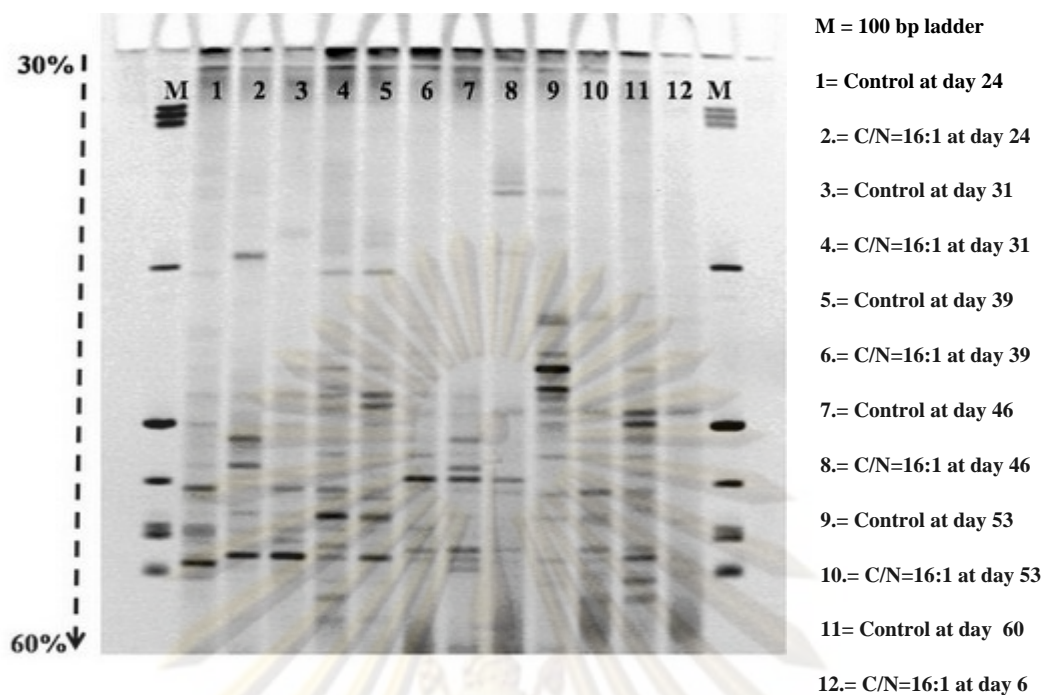
DGGE profile of 16S rRNA gene amplified with primers PRBA338f+GC clamp and PRUN518r.

Figure 4.15 Result of PCR-DGGE analysis of biofloc samples from the controls (feed only) and treatments (C:N = 16:1)

Table 4.7 Result of proximate analysis for carbon, hydrogen, and nitrogen (CHN) contents of biofloc from the controls and treatments

Day	Carbon (% dried wt)		Hydrogen (% dried wt)		Nitrogen (% dried wt)	
	Control	Treatment	Control	Treatment	Control	Treatment
24	24.75	33.53	3.90	4.86	2.05	3.68
31	23.25	35.19	3.42	4.52	2.34	4.56
46	17.48	35.42	5.23	4.88	1.84	4.57
60	21.34	33.91	3.53	4.49	2.53	4.16
Average	21.71±3.14[a]	34.51±0.93[b]	4.02±0.83	4.69±0.21	2.19±0.31[a]	4.24±0.42[b]
T-Test	$P = 0.0033$		$P = 0.0725$		$P = 0.0022$	

[a,b] Indicate statistically significant differences ($P < 0.05$)

Table 4.8 The nitrogen balance in the controls (feed only) and the treatments (C:N = 16:1) calculated after experiment concluded on day 60

	Control (N-Only)		Treatment (C:N=16:1)	
	Nitrogen (g/tank)	% Nitrogen	Nitrogen (g/tank)	% Nitrogen
Input				
Total (feed)	76.19	100	286.07	100
Output				
DIN	33.21	43.59	54.66	19.11
Biofloc	3.02	3.96	26.73	9.34
Tilapia	34.26	44.97	85.29	29.81
Others	5.70	7.48	119.39	41.74
Total	76.19	100	286.07	100

Oxygen Consumption
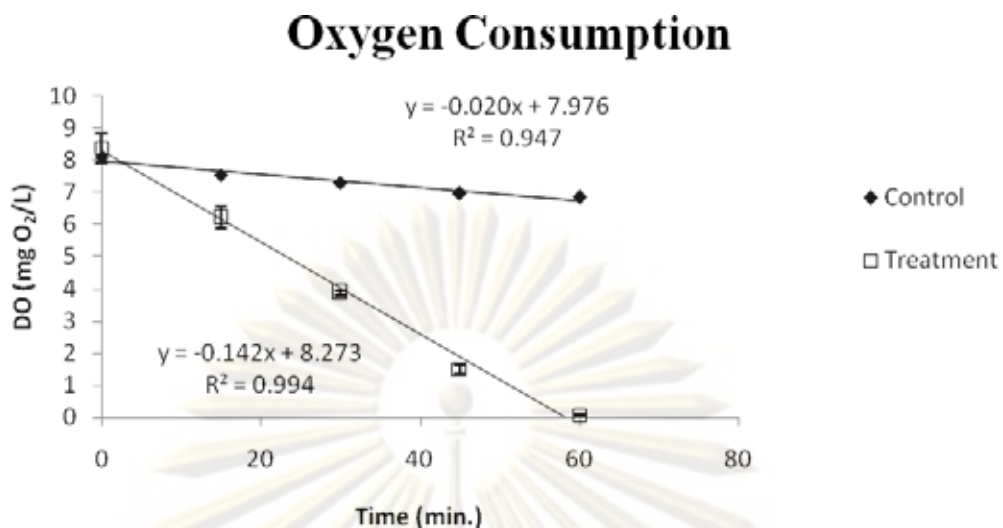
Figure 4.16 Result from an independent experiment to determine the oxygen consumption rate of biofloc from the controls (feed only) and treatments (C:N = 16:1).

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. Organic carbon addition at high C:N ratios was more effective in maintaining the TAN and nitrite concentrations in comparison to lower C:N ratios. Based on the results obtained during the first part from this work, the daily addition of carbon and nitrogen at C:N ratio of 16:1 provided the most effective inorganic nitrogen controls despite reporting a high concentration of particulate matters at approximately 750 mg SS/L. Direct assimilation of inorganic nitrogen by phytoplankton and heterotrophic bacteria was believed to be the main mechanisms that were responsible for the effective nitrogen removal efficiency obtained. The occurrence of nitrification also exerted the positive impact on water quality as it converted toxic ammonia into nitrate. Finally, the coexistence of microalgae, zooplankton, nitrifying bacteria and heterotrophic bacteria suggested the complex ecological relationships established within the biofloc. In addition, the use of pond water containing natural bioflocs appeared more effective than tap water in controlling inorganic nitrogen.

2. For the second part of the work, the daily addition of C:N ratio at 16:1 was more effective in maintaining TAN and nitrite in water. Despite a significant increase of suspended solids from 30 to 1,118 mg SS/L, the effective nitrogen treatment did not proceed until a complete nitrification was established in the tanks, thereby implying that the water must be pre-acclimated to achieve the complete nitrification before being used in biofloc systems. The morphology of bioflocs was similar to those observed in the first section: the biofloc structure was irregular shape containing filamentous microorganisms, rotifers, nematode, and small amount of microalgae. The C, H and N analysis revealed that the carbon and nitrogen contents of bioflocs in the treatments were at 34.5% and 4.2%, respectively whereas the carbon and nitrogen contents in the

controls were at 21.7% and 2.19%. The nitrogen balance and PCR-DGGE analysis indicated that biofloc were highly diverse and dynamics. In term of system management, the addition of organic carbon compounds at high C:N ratio could be employed as a quick solution to reduce the excessive levels of TAN and nitrite in water. Biofloc systems required intensive oxygen supply to maintain the carbon and nitrogen degradation. Malfuction of aeration equipment in intensive aquaculture systems could lead to a rapid decrease of DO concentration to a critical level (i.e., DO < 1.0 mg O_2/L) within an hour.

5.2 Recommendation and Contributions

The result from this work suggested the possibility of adopting the biofloc technology systems in Thailand. The consequence of this work may be useful to an initiation of research activities related to the biofloc technology in Thailand. Successful operation of biofloc technology would assist particularly the tilapia and shrimp farmers to reduce the feed expense and elevate the farming standards to meet in environmental regulations and good production practices. Future research should focus on identifying the microorganisms that are responsible for the phenomena occurred in biofloc technology ponds. Optimization of the system under larger scale should also be carried out and accompanied by an economical evaluation. Additional studies on the value added of biofloc should be investigated in the future.

REFERENCES

Abdel-Fattah, A. and El-Sayed, M., 2006. Tilapia Culture. UK: CABI Publishing.

Al-hafedh, Y.S., Alam, A., Alam, M.A., 2003. Performance of plastic biofilter media with different configuration in water recirculating system for the culture of Nile tilapia *(Oreochromis niloticus)*. Aquacultural Engineering 29, 139-154.

Anderson, G.K. and Ibrahim, A.B., 1978. Treatment of High Nitrate Wastewater by Plastic Media Anaerobic Filters with Particular Reference to Latex Processing. Progress Water Technology 10, 237-253.

APHA. American Public Health Association, 1998. Standard methods for the examination of water and wastewater. Maryland: Victor Graphics.

Avnimelech, Y., kochva, M. and Diab, S., 1994. Development of controlled intensive aquaculture systems with a limited water exchange and adjusted carbon to nitrogen ratio. Israeli Journal of Aquaculture-Bamidgeh 46(3), 119-131.

Avnimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176, 227-235.

Avnimelech, Y. and Ritvo, G., 2003. Shrimp and fish pond soils: processes and management. Aquaculture 220, 549-567.

Avnimelech, Y., Ritvo, G., Kochva, M., 2004. Evaluating the active redox and organic fraction in pond bottom soils: EOM, easily oxidized material. Aquaculture 233, 283-292.

Avnimelech, Y., 2006. Bio-filters: The need for a new comprehensive approach. Aquacultural Engineering 34, 172-178.

Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture 264, 140-147.

Avnimelech, Y., and Benjarano, I., 2007. Probiotic effects of bio-floc technology: depression of streptococci infection of tilapia. Available from <http://floc.aesweb.org/library/sanantonio2007/avinemelech.pdf>

Avnimelech, Y., van Wyk, P., 2007. Biofloc technology: principle and application. Presentation Aquaculture 2007, San Antonio, Texas USA. Feb 26 – Mar 2.

Azim, M.E., Little, D.C., Bron, J.E., 2008. Microbial protein production in activated suspension tanks manipulating C:N ratio in feed and the implication for fish culture. Bioresource Technology 99(9), 3590-3599.

Balarin, J.D. and Haller, J.P., 1979. Tilapia a guide to their Biology and Culture in Africa. University of Stirling, Stirling, Scotland.

Bache, D. H., Johnson, C., McGilligan, J. F. and Rasool, E., 1997. A conceptual view of floc structure in the sweep floc domain. Water Science and Technology 36(4), 49 56.

Biggs, C.A. and Lant, P.A., 2000. Activated sludge flocculation: on-line determination of floc size and the effect of shear. Water Research 34(9), 2542-2550.

Boyd, C.E. and Tucker, C.S., 1998. Pond aquaculture water quality management. Norwell, Massachusetts: Kluwer Acadamic Publishers.

Boyd, C.E. and Clay, J., 2002. Evaluation of Belize Aquaculture Ltd: A superintensive shrimp aquaculture system, A report prepared under the World Bank, NACA, WWF, and FAO Consortium Program on Shrimp Farming and the Environment.

Bitton, G., 1994. Wastewater microbiology. New York, USA: John Wiley& Son.

Brazil, B.L., 2006. Performance and operation of rotating biological contactor in a tilapia recirculating aquaculture system. Aquaculture Engineering 34, 261-274.

Brune, D.E., Schwartz, G., Eversole, A.G., Collier, J.A. and Schwedler, T.E., 2003. Intensification of pond aquaculture and high rate photosynthesis system. Aquacultural Engineering 28, 65-86.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., and Pearson, D.C., 2003. Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. Aquaculture 219, 393-411.

Burford, M.A. and Lorenzen, K., 2004. Modeling nitrogen dynamic in intensive shrimp ponds: The role of sediment reminerlization. Aquaculture 219, 393-411.

Christensen, H.M. and Harremoes, P., 1977. Biological denitrification of sewage literature review. Progress Water technology 8, 509-556.

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, B. and Verstraete, W., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270(1-4), 1-14.

Cruz, E.M., Ridha, M.T., 1995. Survival rates of tilapia, *Oreochromis spilurus* (Gunter), fingerlings reared at high densities during winter using warm Underground sea water. Aquaculture Research 26, 307-309.

DeBoer, J.A. Nutrients. In. C.S. Lobban and M.J. Wynne (eds)., 1981. The biology of seaweeds. Berkely: University of California Press.

Decamp, O., Cody, J., Conquest, L., Delanoy, G., Tacon, A.G.J., 2003. Effect of salinity on natural community and production of *Litopenaeus vannami* (Boone), with experimental zero water exchange culture systems. Aquaculture Research 34, 345- 355.

Defoirdt, T., Halet, D., Vervaeren, H., Boon., N., van de Wiele, T., Sorgeloos, P., Bossier., P. and Verstraete, W., 2007. The bacterial storage compound poly-β hydroxybutyrate protects Artemia franciscana from pathogenic *Vibrio campbellii*. Environmental Microbiology 9, 149-169.

Delos Reyae, A.A. and Lawson T.B., 1996. Combination of a bead filter and rotating biological contactor in a circulating fish culture system. Aquacultural Engineering 15, 27-39.

De Schryver, P., Crab, R., Defoirdt, T., Boon, N., Verstraete, W., 2008. The basics of bio-flocs technology: The added value for aquaculture. Aquaculture 277, 125-137.

Ebeling, J.M. and Timmons, M.B., 2007. Stoichiometry of ammonia-nitrogen removal in zero-exchange systems. World Aquaculture 38(2), 22-25.

Eding, E.H., Kamstra, A., Verreth, J.A.J., Huisman, E.A. and Klapwijk, A., 2006. Design and operation of nitrifying trickling filters in recirculting aquaculture: a review. Aquaculture Engineering 34, 234-260.

FAO., 2004. State of World Fisheries and Aquaculture. FAO, Room. Flemish government, 2005. Afvalwaterproblematiek op melkveebedrij-ven. Flemish Government, Department Agriculture and Fisheries, Department Sustainable Agriculture Development, Belgium, 61 pp.

Feray, C. and Montuelle, B., 2003. Chemical and microbial hypotheses explaining the effect of wastewater treatment plant discharges on the nitrifying communities in freshwater sediment. Chemosphere 50, 919-928.

Flemming, H. C. and Wingender, J., 2001. Relevance of microbial extracellular polymeric substances (EPSs) – Part I: Structural and ecological aspects. <u>Water Science and Technology</u> 43(6), 1-8.

Fontenot, Q., Bonvillain, C, Kilgen, M. and Boopathy, R., 2007. Effects of temperature,salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. <u>Bioresource Technology</u> 98, 1700-1703.

Gaudy, A.F., Jr., Gaudy, E.T., 1980. <u>Microbiology for Environmental Scientists and Engineers.</u> McGraw-Hill, New York, 736 pp.

Greiner, A.D. and Timmons, M.B., 1998. Evaluation of nitrification rate of Microbead and trickling filters in an intensive recirculating tilapia production facility. <u>Aquaculture Engineering</u> 18, 189-200.

Gross, M., Boyd, C.E., Seo, J., 1999. Evaluation of the Ultraviolet Spectrophotometric Method for the Measurement of Total Nitrogen in Water. <u>World Aquaculture Society</u> 30, 389-393.

Gutierrez-Wing, M.T. and Malone, R.F., 2006. Biological filters in aquaculture: trends and research directions for freshwater and marine applications. <u>Aquaculture Engineering</u> 34 (3), 163-171.

Hargreaves, J.A., 1998. Nitrogen biogeochemistry of aquaculture ponds. <u>Aquaculture</u> 166, 181-212.

Hargreaves, J. A., 2006. Photosynthesis suspended-growth systems in aquaculture. <u>Aquaculture engineering</u> 34, 344-363.

Hari, B., Kurup, B.M., Verghese, J.T., Schrama, J.W., Verdegem, M.C.J., 2004. Effect of carbohydrate addition on production in extensive shrimp culture systems. <u>Aquaculture</u> 241, 179-194.

Hari, B., Kurup, B.M., Varghese, J.T., Schrama, J.W. and Verdegem, M.C.J., 2006. The effect of carbohydrate addition on water quality and the nitrogen budget in extensive shrimp culture systems. Aquaculture 252, 248-263.

Khin, T. and Annachhatre, A.P., 2004. Novel microbial nitrogen removal processes. Biotechnology Advances 22, 519-532.

Knowler, R., 1982. Denitrification. Microbiological review 46, 43-70.

Krishna, C. and Van Loosdrecht, M.C.M., 1999. Effect of temperature on storage polymera and settleability of activated sludge. Water Research 33 (10), 2374-2382.

Lekang, O.I. and Kleppe, H., 2002. Efficiency of nitrification in trickling filters using difference filter media. Aquacultural Engineering 21, 181-199.

Lehr, J. and Keeley, J., 2005. Water Encyclopedia. Vol. 1-5. USA: John Wiley&Sons.

Little, D.C., Murray, j.F., Azim, M.E., Leschen, W., Grady, K., Young, j., Watterson, A., 2008. Warm-water fish production in the UK: limits to green growth? Trends in Food Science and Technology 19, 255-264.

Logan, B.E., and Hunt, J.R., 1988. Bioflocculation as microbial response to substrate limitations. Biotechnology and Bioengineering 31, 91-101.

Losordo, T.M., Westerman, P.W., 1994. An analysis of biological, economic, and engineering factors affecting the cost of fish production in recirculating aquaculture systems. Journal Worlds Aquaculture Society 25, 1193-203.

Lu, F., Wendell, H.C., Chian, E.S.K., Pohland, F.G. and Gas, H., 1984. Nitrification of anaerobically treated coal gasifier effluent. Journal of water Pollution Control Federation 56 (9), 1050-1058.

Martin, A. M. P., Pagilla, K., Heijnen, J. J., and van Loosdrecht, M. C. M., 2004.
Filamentous Bulking Sludge – A Critical Review. Water Research 38, 793-817.

Matos, J., Costa, S., Rodrigues, A., Pereira, R. and Pinto, i.S., 2006. Experimental
integrated aquaculture of fish and red seaweeds in Northern Portugal. Aquaculture
252 (1), 31-42.

McGraw, W., Teichert-Coddington, D.R., Rouse, D.B., Boyd, C.E., 2001. Higher
minimum dissolved oxygen concentrations increase penaeid shrimp yields in
earthen ponds. Aquaculture 199, 311-321.

McIntosh, R.P., 2001. High rate bacterial system for culturing shrimp. Proceedings
from aquacultural engineering society's second issues forum. Ithaca. NY, NRAES
157, 117-129.

Mohammad, T.R. and Emmanuel, M.C., 2000. Effect of biofilter media on water
quality and biological performance of the Nile tilapia *Oreochromis niloticus* L.
reared in a simple recirculating system. Aquacultural Engineering 24, 157-166.

Moss, S.M., Arce, S.M., Argue, B.J., Otoshi, C.A., Calderon, G.R.O., Tacon, A.G.J.,
2001. Greening of the blue revolution: efforts toward environmentally responsible
shrimp culture. In: Browdy, C.L., Jory, D.E. (Eds.), The New Wave, Proceedings
of the Special Session on Sustainable Shrimp Culture, Aquaculture 2001, World
Aquaculture Society, Baton Rouge, LA, USA, pp.1-19.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G., 1993. Profiling of complex
microbial populations by denaturing gradient gel electrophoresis analysis of
polymerease chain reaction-amplified genes coding for 16SrRNA. Applied and
Environmental Microbiology 59, 659-700.

Muyzer, G. and Smalla, K., 1998. Application of denaturing gradient gel
electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in
microbial ecology. Antonie van Leeuwenhoek 73, 127-141.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.

Nootong, K., 2006. Performance and kinetic evaluation of a novel bioreactor system in the low-oxygen/low-fluid shear reaction environment. P.h.D., Thesis, University of Pennsylvania, Philadelphia, PA, USA.

Nootong, K., 2008. Nitrogen Treatment in Closed-System Aquaculture. KMITL Science and Technology Journal 16, 11-22.

Nootong, K. and Shieh, W.K., 2008. Analysis of an upflow bioreactor system for nitrogen removal via autotrophic nitrification and denitrification. Bioresource Technology 99 (14), 6292-6298.

Panjaitan, P., 2004. Field and laboratory study of *Penaeus monodon* culture with zero water exchange and limited water exchange model using molasses as a carbon source. Ph.D. thesis, Charles Darwin University, Darwin, NT, Australia.

Ross, L.G., 2000. Environmental physiology and enertics. In: Berveridge, M.C.M. and McAndrew, B.J.(eds.). Tilapia biology and Exploitation. Dordrecht/ Boston/ London: Kluwer Acadamic Publishers.

Sales, C.M. and Shieh, W.K., 2006. Performance of anaerobic/anaerobic hybrid bioreactor under the nitrogen deficient and low F/M conditions. Water Research 40, 1442-1448.

Sandu, S.I., Boardman, G.D., Watten, B.J., and Brazil, B.L., 2002. Factor influencing the nitrification efficiency of fludized bed filter with plastic bead medium. Aquaculture Engineering 26, 41-59.

Sastry, B.N., Delos Reyes Jr., A. A., Rusch, K.A. and Malone, R.F., 1999. Nitrification performance of a bubble-washed bead filter for a combined solids removal and biological filtration in a recirculating aquaculture system. Aquacultural Engineering 19, 105-117.

Sato, C., Leung, S.W., and Schnoor, L.J., 1988. Toxic response of *Nitrosomonas europaea* to copper in inorganic medium and wastewater. Water research 22, 1117 1127.

Schryver, P.D. and Verstraete, W., 2008. Nitrogen removal from aquaculture pond water by heterotrophic nitrogen assimilation in lab-scale sequencing batch reactors. Bioresouece Technology 100, 1162-1167.

Schurr, A.M., 2003. Evaluation of biosecurity application for intensive shrimp farming. Aquacultural Engineering 28, 3-20.

Sharma, B. and Ahler, R.C., 1977. Nitrification and nitrogen removal. Water Research 11, 897-925.

Shieh, W.K. and Keenan, J.D., 1987. Fluidized Bed Biofilm Reactor for Wastewater Treatment, in: Fiechter, A., (Eds). Advances in Biochemical Engineering /Biotechnology 33, 131-169.

Strickland, J.D.H. and Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis. (second edition), 185-192.

Subasinghe, R.P., 2005. Epidemiological approach to aquatic animal health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. Preventive Veterinary Medicine, 67 (2-3), 117-124.

Summerfelt, S.T. and Sharrer, M.J., 2004. Design implication of carbon dioxide production within biofilters contained in recirculating salmonid culture system. Aquaculture Engineering 32, 171-182.

Tacon, A.G.J., Cody, J., Conquest, L., Divakaran, S., Forster, I.P. and Decamp, O., 2002. Effect of culture system on the nutrition and growth performance of Pacific white shrimp *Litopenaeus vannami* (Boone) fed different diets. Aquaculture Nutrition 8, 121-137.

Taoka, Y., Maeda, H., Jo, Jae-Yoon., Kim, Su-mi., Park, So-li., Yoshikawa, T. and Sakata, T., 2006. Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. Fisheries Science 72, 755-766.

Tchobanoglous, G. and Burton, F.L., 2004. Wastewater Engineering: treatment, disposal and reuse, 4rd ed. McGraw-Hill, New York, U.S.A.

Thakur, D.P., Lin, C.K., 2003. Water quality and nutrient budget in closed shrimp (*Penaeus monodon*) culture systems. Aquaculture Engineering 27, 159-176. U.S. EPA., 1975. Process Design Manual for Nitrogen Control. Washington D.C.: Office of technology Transfer.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., and Vinci, B.J., 2002. Recirculating aquaculture system, 2nd ed. NRAC Publication, New York, USA.

Triyarat, W., 2003. Optimization of nitrate treatment system for marine shrimp culture tank using the tubular denitrification reactor. Master of Science Thesis in Environment Science (Inter-department). Graduate School Chulalongkorn University, Bangkok, Thailand.

Twarowska, J.G., Westerman, P.W. and Losordo, P.M., 1997. Water treatment and waste characterization evaluation of an intensive fish production system. Aquacultural Engineering 16, 133-147.

U.S. EPA., 1975. Process Design Manual for Nitrogen Control. Washington D.C.: Office of technology Transfer.

Vanitchania, W., Nootong, K., 2008. Effects of organic carbon addition in controlling inorganic nitrogen toxicity for the closed-water aquaculture applications. Proceeding to the 34th congress on science and technology of Thailand, Queen Sirikit National Convention Center, Bangkok, Thailand, Oct. 31- Nov 2.

Van Rijin, J., 1996. The potential for integral biological treatment system in recirculating culture review. Aquaculture 139, 181-201.

Verstraete, W., Alexander, M., 1973. Heterotrophic nitrification in samples of natural ecosystems. Environment Science and technology 7, 39-42.

Verstraete, W., De Schryver, P., Defoirdt, T., Crab, R., 2007. Added values of microbial life in flocs. Presentation Aquaculture 2007, San Antonio, Texas USA. Feb 26 – Mar 2, 2007.

Watson, S.W., Valos, F.W., Waterbury, J.B., 1981. The Family Nitrobacteraceae. In: The Prokaryotes. Spinger-Verlag, Berlin.

Wilen, B.M. and Balmer, P., 1999. The effect of dissolved oxygen concentration on the structure size, size distribution of activated sludge floc. Water Research, 33(2), 391-400.

Winkler, M.A., 1984. Fixed-film biological processes for wastewater treatment. The Chemical Engineering Journal 28, 62-63.

Wutikumpoln, M., 2003. Effect of biofilter on water quality in TABTIM fish *Oreochromis niloticus* close culture pond. Master of Science Thesis in Environment Science (Inter-department). Graduate School Chulalongkorn University, Bangkok, Thailand.

APPENDICES

Appendix A

Water quality analysis

A-1 Analysis of physical factors

A-1.1Dissolved oxygen (DO)

Dissolved oxygen (DO) (mg O_2/L) was monitored using by DO meter (HI91410, Hanna).

A-1.2 Temperature

The water temperatures are measured by logging pH meter (YSI, pH10).

A-1.3 pH

The pH water are measure by pH meter method (YSI, pH10).

A-1.4 Floc volume index (FVI)

FVI was determined by sampling 1000 ml pond water into a series of Imhoff cones (Eaton et al. 1995). The volume of the floc plug accumulating on the bottom of the cone was determined 30 min following sampling.

A-2 Determination of Total ammonia nitrogen Concentration

Apply the sample through Whatmann GF/C filter paper. Collect the clear liquid sample that added 0.2 mL phenol solution (dissolve 20 g phenol in 200 mL of 95% v/v ethy alcohol) into 5 mL sample, and mix with 0.2 mL sodium nitroprusside solution (dissolve 1.0 g of sodium nitroprusside into 200 mL of deionized water), and 0.5 mL oxidizing solution, which is prepared by dissolving 100 g of sodium citrate and 5.0 g of sodium hydroxide in 500 ml of deionized water and 25 mL of sodium hypochlorite solution. Keep the oxidizing solution stoppered while it is not in use. Read the absorption at the 640 nm wavelength by a spectrophotometer.

The preparation of standard ammonia solution that dissolved 0.1 g of analytic grade ammonia sulfate in 1,000 ml distilled water and store sheltered from strong light. The solution is stable for many months afterward. Prepare 0.1, 0.2, 0.5, 1.0 and 2.0 mg N/L.
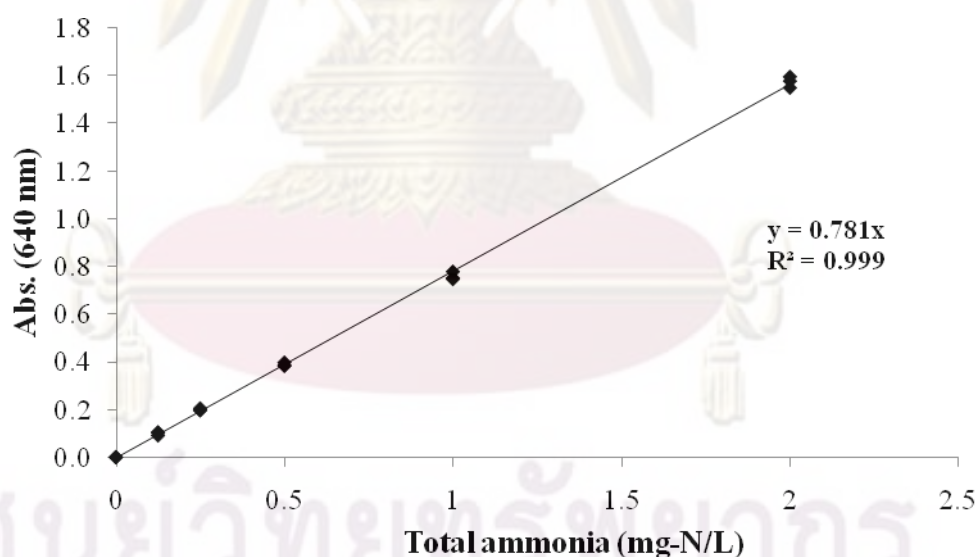
Figure A-1 Standard curve of total ammonia concentration (NH_4^+-N and NH_3-N)

A-3 Determine of Nitrite Concentrations

Apply the sample through Whatmann GF/C filter paper. Collect the clear liquid sample. Samples are stable in subdued light for many hours at room temperature but the analysis should not be delayed for more than about 5 – 10 hours. If greater delays are unavoidable, the samples should be frozen. The blank is prepared by using 5 mL of distilled water. Then, mix 5 ml of the sample with 0.1 ml sulphaniamide (dissolve 5 g of sulphaniamide in 50 ml of concentrated HCl and 3,000 ml of distilled water) and allow the reagent to react for 2 – 8 minutes. Added 0.1 ml naphthyethylenediamine solution (dissolve 0.5 g of dihydrochloride in 500 ml distilled water) into the sample, mix immediately and allow 10 minutes reaction period. Read the absorption at the 543 nm wavelength by using spectrophotometer.

The preparation of standard nitrite solution that dissolved 0.345 g of analytical grade sodium nitrite in 1,000 ml distilled water and store in a dark bottle. The solution is stable for at least 1 – 2 months. Prepare the nitrite solution at 0.05, 0.1, 0.2, 0.4 and 0.8 mg N/L.

$$y = 3.241x$$
$$R^2 = 0.999$$
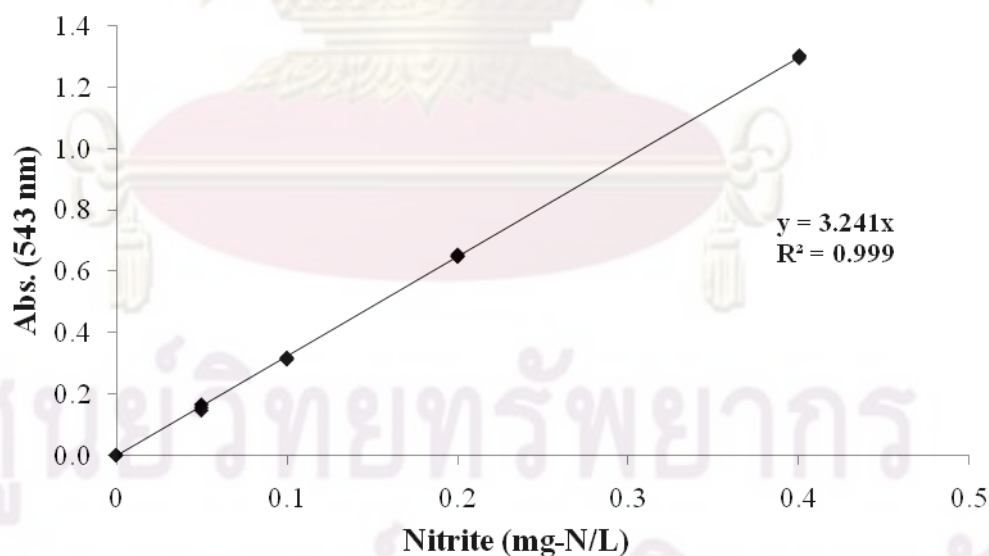
Figure A-2 Standard curve of nitrite (NO_2^--N)

A-4 Determine of Nitrate Concentrations

Apply the sample through Whatmann GF/C filter paper. Collect the clear liquid sample. The analysis should be performed no longer than 10 hours or it is necessary to freeze the sample for storage. The blank is prepared by using 5 ml of distilled water. The nitrate concentration will be measured by the spectrophotometer based on the absorption at the wavelength 220 nm and 275 nm.

The preparation of standard nitrate that dissolved 1.02 g of analytical grade potassium nitrate in 1,000 ml distilled water and store in a dark bottle.   Prepare the nitrate solution at 0.625, 1.25, 2.5, 5 mg N/L. The calculation of nitrate concentration was as following:

$$Nitrate\ (mg - N/L) = \frac{(Abs_{220\ nm} - Abs_{275nm}) \times A}{B}$$

Where A = concentration of nitrate in standard curve (mg-N/L)

B = absorbance of standard curve (220-275 nm)

Standard nitrate solution was prepared using 1-10 mg-N/L of sodium nitrate. It has to be note that, this method must be strictly used with nitrate concentration between 1-10 mg-N/L. Water sample containing high nitrate concentration (over 10 mg-N/L) can be diluted with de-ionized water prior to analysis but water containing low nitrate concentration (below 1 mg-N/L) was not applicable with this method. Moreover, high nitrite concentration can interfere with nitrate measurement hence concentration of nitrate must be subtracted with nitrite concentration.
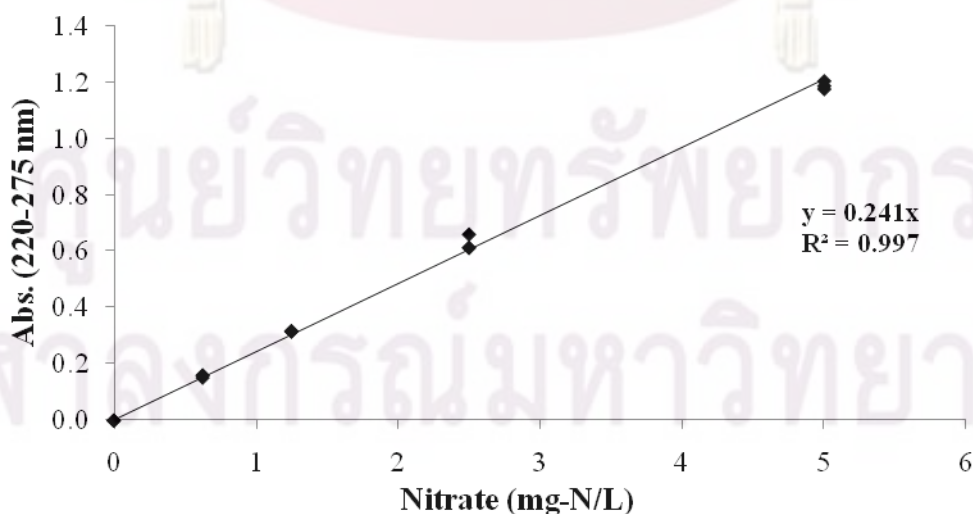
Figure A-3 Standard curve of nitrate (NO_3^--N)

A-5 Total Solids (TS)

Obtain a dried crucible and weigh to obtain its dried weight. Apply a known volume of liquid sample containing solids into the dried crucible. Put the crucible in the controlled temperature oven overnight at 103 – 105 °C. After a night, bring the crucible containing the dried residues out of the oven and cool in a desicator for at least 30 minutes. Weigh the crucible. The concentration of total solids is:

$$\text{Total solids (mg/L)} = \left(\frac{(A - B) \times 1000}{C} \right)$$

Where A is the weight of crucible and dried residue, mg; B is the weight of dried crucible, mg; and C is the volume of filtered water, ml

A-6 Total Suspended Solids (TSS)

Dry the Whatmann GF/C filter (47 mm) in an oven overnight at 103 – 105 °C. Store the dried filter in desiccators. Weigh the dried filter. Attach the dried filter to the filtering apparatus, which is connected to the vacuum pump. Pour a known volume of liquid containing suspended solids through a filtering apparatus. The residue retained on the filter paper is dried in the temperature controlled oven overnight at 103 – 105 °C to a constant weight. After a night, bring the filter out of the oven and cool in desiccators for at least 30 minutes. Weigh the filter paper.

The concentration of suspended solid is:

$$\text{Total suspended solids (mg/L)} = \left(\frac{(A - B) \times 1000}{C} \right)$$

Where A is the weight of filter paper plus dried residue, mg; B is the weight of filter paper, mg; and C is the volume of filtered water, ml

A-7 Determine of Chlorophyll Concentrations

Apply 100 mL sample through 25 mm Whatmann filter paper. If immediate analysis cannot be done, the filter sample must be frozen and stored as long as 2 – 3 weeks. The chlorophyll retained on the filtered paper is extracted by using 5 mL of 90% (v/v) acetone under subdued light condition. The sample is stored at 4 °C in the refrigerator for 24 hours to avoid chlorophyll degradation. The filter paper is placed in the tube, which is flooded with 5 mL of 90% (v/v) acetone. The tubes were shaken before centrifuged at 4000 rpm for 20 minutes. The absorption of pigment extracted is determined by using the spectrophotometer at 630, 645 and 665 nm wavelength. The calculation of chlorophyll concentrations can be done by the following formulas.

$$C_a(mg/m^3) = \frac{(11.6D_{665} - 1.31D_{645} - 0.14D_{630}) \times v}{l \times V}$$

$$C_b(mg/m^3) = \frac{(20.7D_{645} - 4.34D_{665} - 4.42D_{630}) \times v}{l \times V}$$

$$C_c(mg/m^3) = \frac{(55D_{630} - 4.64D_{665} - 16.3D_{645}) \times v}{l \times V}$$

Where: D_{630}, D_{645} and D_{665} are the optical density at 630 nm, 645 nm and 665 nm wavelength, respectively; v is volume of 90% acetone, ml, V = volume of filtered water, L; l is the cuvette cell length, cm.

A-8 Total nitrogen (TN)

TN of water sample was modified from Gross et al 1999. A 2.5 ml unfiltered water sample was added with 1.25 ml of oxidize reagent then the sample was covered by foil, bind of plastic bland and autoclave at 121°C for 30 minutes. After cooling the room temperature and added 0.25 ml of borate buffer, mixing and centrifuges for 5 minutes that can be measured by nitrate nitrogen method that can be found in A-4. The oxidizing solution can be prepared by 0.75 g of sodium hydroxide (NaOH) and 0.1 g of potassium persulfate (K_2SO_3) in 250 ml of de-ionized water and borate buffer can be prepared by 15.45 g of boric acid (H_3BO_3) and 2 g of sodium hydroxide (NaOH) in 250 ml of de-ionized water.

A-9 Dissolved total nitrogen (DTN)

DTN of water sample was modified from Gross et al 1999. A 2.5 ml filtered water sample was added with 1.25 ml of oxidize reagent then the sample was covered by foil, bind of plastic bland and autoclave at $121^{o}C$ for 30 minutes. After cooling the room temperature and added 0.25 ml of borate buffer, mixing and centrifuges for 5 minutes that can be measured by nitrate nitrogen method that can be found in A-4. The oxidizing solution can be prepared by 0.75 g of sodium hydroxide (NaOH) and 0.1 g of potassium persulfate (K_2SO_3) in 250 ml of de-ionized water and borate buffer can be prepared by 15.45 g of boric acid (H_3BO_3) and 2 g of sodium hydroxide (NaOH) in 250 ml of de-ionized water.

A-10 Determine of nitrification potential

Potential of nitrification was determined by using modified method from Feray Montuelle, 2003.

1. The chemical reagent for nitrification potential

- Ammonium sulphate ($(NH_4)_2SO_4$)

- Sulphuric acid (H_2SO_4)

- Sodium hydroxide (NaOH)

- N-allythiourea (ATU)

- Sodium nitrite ($NaNO_2$)

- Sodiumbicarbonate ($NaHCO_3$)

- Sodium chlorate ($NaClO_3$)

2. The rate of nitrification

$$Control : NH_3 \rightarrow NO_2 \rightarrow NO_3 \quad Nitrification$$

$$ATU : NH_3 \nrightarrow NO_2 \downarrow \rightarrow NO_3 \uparrow \quad Nitrite\ oxidizing$$

$$NaClO_3 : NH_3 \downarrow \rightarrow NO_2 \uparrow \nrightarrow NO_3 \quad Ammonia\ oxidizing$$
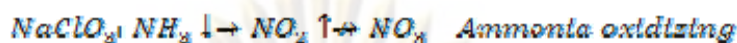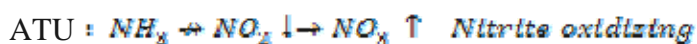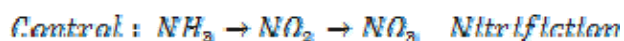
The rate of nitrification potential in water sample was determined by 50 ml of water sample in flask. Control in the first flask did not receiving any inhibitor (ATU and NaClO$_3$) as nitrification that added with 6.25 ml with NaHCO$_3$ and adjust pH in between 7-8 with 20% H$_2$SO$_4$ or 10% NaOH. Second flask was nitrite oxidizing activity, added with 6.25 ml of NaHCO$_3$ and 0.625 ml of ATU. Then filled NaNO$_2$ concentration at 0.5, 1, 2, 5, 10 mg-N/L in each flask for nitrite oxidizing respectively and adjust pH in between 7-8 with 20% H$_2$SO$_4$ or 10% NaOH. The third flask was ammonia oxidizing activity, added with 6.25 ml of NaHCO$_3$ and 0.625 ml of NaClO$_3$. Then filled (NH$_4$)$_2$SO$_4$ concentration at 0.5, 1, 2, 5, 10 mg-N/L in each flask for ammonia oxidizing respectively and adjust pH in between 7-8 with 20% H$_2$SO$_4$ or 10% NaOH. All flasks were incubated by shaken at the 120 rpm, at 25°C, and under dark condition then keep the each sample at the beginning 0, 2, 4 and 8 respectively. All flask was to determine nitrite concentration which determined by nitrite nitrogen concentration can be found in section A-2.

Appendix B

Molecular analysis

B-1 PCR and DGGE analysis for assessment bacteria community

1. The materials for analytical bacteria community

Materials	Trademark
1.Test kit for extracted DNA (Fast DNA spin kit from soil)	QBiogene, Solon, Ohio, USA
2.Vortex	Scientific Industries, Inc. (Vortex-Genie 2)
3.Shaker	Thermo Electron Corporation (Fast PREPTM FP 120)
4.Centrifuge	Eppendrof (5804R)
5.Temperature controller for PCR	Thermo Electron Corporation (HB-PX-2220)
6.DNA Electrophoresis Gel Boxs	Bioactive, Inc
7.DGGE (D code system)	Bio-Rad laboratories, Inc
8.UV Transilluminator Gel	Wealtec (Gel Dolphic-DOC)

2. The chemical reagent for analytical bacteria community

2.1 Chemicals for extracted DNA

1. Sodium phosphate buffer

2. MT buffer

3. PPS reagent

4. Binding Matrix Suspension

5. SEWS-M

6. DES

2.2 Chemicals for PCR

1. 10X PCR buffer

2. dNTP mix

3. Primer CTO 189A/Bf-GC

4. Primer CTO 189Cf-GC

5. Primer CTO 654r

6. Tag DNA polymerase

7. Distilled water

8. Template DNA

2.3 Chemicals for DGGE

1. 40% Acrylamide

2. 50X TAE

3. Formamide

4. Urea

5. APS

6. TEMED

7. Dye solution

3. DNA extract and 16s rDNA amplification from suspended floc

The 5 ml of water sample was centrifuge at 1000 rpm for 10 minutes or bacteria were collected by centrifuge at 1200 rpm for 5 minutes. Supernatant was removed and bacterial pellet was stored in the freezer at -20°C for further molecular analysis. Genomic DNA was extracted using Chelex 100 resin (Bio-Rad laboratories, USA). Bacterial pellet were mixed with 50 µL which Chelex 100 resin solution was prepared from 0.1 mg of resin in 1 ml final volume of 1xTE buffer, centrifuged at 2500 rpm for 5 minutes, incubated at 56°C for 1 hour with gently shaking and the further incubated at 95°C for 15 minutes. After extraction process, the extracted DNA solution was stored at 4°C for period to use. Then, the PCR amplification of 16s rRNA gene can be used by PRBA338f-GC clamp and PRUN 518 r primers. As the result, the separation of PCR products can be used by Denaturing Gradient Electrophoresis (DGGE).

Table B-1.1 Show the PCR condition and PCR mixtures for the DNA extraction suspended floc

PCR condition		PCR mixtures	
Initial denaturation	94°C, 2 min	DNA	5 µl
Denaturation	94°C, 1 min	dH$_2$O	7 µl
Annealing	55°C, 30 sec	PRBA 338f-GC clamp (10 5 µM)	1.5 µl
Extension	72°C, 1 min	PRUN 518r (10 µM)	1.5 µl
Final extension	72°C, 6 min	Taq PCR Master Mix kit (5U/1.5 µl)	15 µl

Table B-1.2 Target sites, sequence and specificity

Primer	Target site	Nucleotide sequence (5´-3´)	Specificity	Expected size	Reference
PRBA338f-GC clamp	338-357	CgC CCg CCg CgC gCg gCg ggC ggg Cgg ggg CAC ggg ggg CCT ACg ggA ggc Agc Ag	All bacteria	236-bp	Muyzer et al. 1993
PRUN518r	518-534	ATT ACC gCg gCT gCT gg	All bacteria		Muyzer et al. 1993

4. Denaturing Gradient Electrophoresis (DGGE) analysis

The 25 µl of PCR products were mixed with 5 µl of 6x loading dye (Bio-Rad Laboratories, USA) then run on 8% of polyacrylamide gradient gel (16x16 cm gel size and 1 mm of gel thick) made by a gradient maker (Bio-Rad Laboratories, USA) according to the manufacture's guidelines. The denaturing gradient range was 30-60% (Muyzer et al. 1998).

The prepare of the gradient gel that can be use 80% denaturing gel solution (20 ml of 37.5:1 acrylamide-bisacrylamide solution, 2 ml of 50x TAE buffer, 33.6 g of urea, 32 ml formamide and adjust volume to 100 ml with dH_2O) was mixed in various proportion with 0% denaturing gel solution (20 ml of 37.5:1 acrylamide-bisacrylamide solution, 2 ml of 50x TAE buffer and adjust volume to 100 ml with dH_2O) in order to obtain the denaturing gradient acrylamide gel with 30 to 60% denaturant. The gel was polymerized by 0.09% of TEMED and 10% of ammonium persulphate. Before polymerization occurred, 3 ml stacking gel without denaturant was added on top of the gradient gel. After DNA loading, DGGE was run at 130 V for 6 hours in 1xTAE buffer at constant temperature ($60^{o}C$). Then, the gel was stained with ethidium bromide solution (dissolved 4 µl of 10 mg/ml ethidium bromide in 50

ml dH$_2$O) for 20 minutes and visualized in gel documentation instrument (Dolphin-Doc Plus, USA).

Appendix C

Data of experiments

Table C-1 Concentration of ammonia, nitrite and nitrate in NH_4Cl only with the glucose as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.00	0.00	0.70	0.00
1	0.02	0.00	0.00	0.00	0.89	0.00
2	11.46	0.37	0.01	0.00	0.84	0.00
3	7.93	0.26	0.03	0.00	0.83	0.00
4	14.95	0.43	0.06	0.00	0.85	0.00
5	18.38	1.07	0.12	0.00	0.88	0.00
6	20.95	0.64	0.18	0.01	1.84	0.00
7	18.29	0.46	0.30	0.01	1.31	0.00
8	17.96	0.42	0.43	0.02	1.59	0.00
9	14.86	0.62	0.56	0.04	1.70	0.00
10	18.73	0.66	0.68	0.02	1.90	0.00
11	11.84	1.27	0.81	0.04	1.68	0.00
12	14.52	0.19	1.04	0.06	2.21	0.00
13	12.36	0.35	1.29	0.04	2.51	0.00
14	12.54	0.38	1.50	0.07	2.61	0.00
15	9.67	0.19	1.52	0.05	3.14	0.00
16	9.99	0.26	1.76	0.03	1.12	0.00
17	11.90	0.26	1.21	0.08	2.89	0.00
18	11.32	0.09	1.11	0.09	2.84	0.00
19	19.96	0.61	1.15	0.07	2.55	0.00
20	9.17	0.56	1.14	0.06	2.77	0.00
21	10.91	0.18	0.83	0.61	2.93	0.00
22	11.64	0.51	1.39	0.08	2.76	0.00
23	10.62	0.24	0.70	0.04	3.16	0.00
24	9.42	0.08	0.90	0.03	3.40	0.00

Table C-2 Concentration of ammonia, nitrite and nitrate in C:N=2:1 with the glucose as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.01	0.01	0.00	0.00	1.15	0.00
1	0.02	0.00	0.01	0.01	0.94	0.00
2	7.66	0.41	0.01	0.00	0.80	0.00
3	7.03	0.47	0.03	0.00	0.81	0.00
4	14.75	0.67	0.05	0.00	0.87	0.00
5	17.59	1.67	0.09	0.00	0.77	0.00
6	19.53	0.10	0.13	0.00	1.20	0.00
7	17.28	0.29	0.21	0.00	1.16	0.00
8	14.66	1.48	0.31	0.00	1.39	0.00
9	12.31	0.50	0.34	0.01	1.74	0.00
10	10.43	1.15	0.50	0.02	1.49	0.00
11	10.18	0.02	0.40	0.01	1.58	0.00
12	11.18	0.50	0.48	0.07	1.66	0.00
13	9.83	0.31	0.60	0.02	1.90	0.00
14	9.31	0.07	0.73	0.01	2.00	0.00
15	8.83	0.04	0.87	0.04	2.41	0.00
16	9.22	0.02	0.96	0.15	1.93	0.00
17	10.89	0.41	0.51	0.03	2.05	0.00
18	9.08	0.13	0.58	0.03	2.24	0.00
19	15.00	0.41	0.57	0.02	1.94	0.00
20	7.68	0.11	0.41	0.04	2.10	0.00
21	7.95	0.33	0.39	0.07	2.19	0.00
22	7.84	0.03	0.55	0.01	2.35	0.00
23	7.98	0.17	0.46	0.04	2.37	0.00
24	6.16	0.26	0.57	0.02	2.44	0.00

Table C-3 Concentration of ammonia, nitrite and nitrate in C:N=4:1 with the glucose as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.00	0.00	0.86	0.00
1	0.01	0.00	0.00	0.00	1.13	0.00
2	15.23	0.09	0.01	0.00	0.87	0.00
3	7.46	0.02	0.03	0.00	0.82	0.00
4	16.18	0.05	0.06	0.00	0.87	0.00
5	18.60	0.75	0.10	0.00	0.83	0.00
6	23.37	0.99	0.13	0.01	1.16	0.00
7	18.89	0.14	0.20	0.01	1.14	0.00
8	17.38	0.84	0.28	0.01	1.31	0.00
9	12.73	0.15	0.32	0.03	1.37	0.00
10	10.96	0.35	0.43	0.01	1.36	0.00
11	11.20	0.31	0.36	0.02	1.39	0.00
12	11.97	0.33	0.33	0.02	1.47	0.00
13	10.23	0.55	0.37	0.02	1.61	0.00
14	9.93	0.01	1.11	0.04	1.63	0.00
15	8.99	0.11	1.22	0.04	1.82	0.00
16	10.25	0.49	1.21	0.07	0.58	0.00
17	11.64	0.24	0.21	0.00	1.64	0.00
18	9.31	0.44	0.49	0.03	1.78	0.00
19	16.49	1.03	0.63	0.04	1.51	0.00
20	7.79	0.12	0.77	0.03	1.81	0.00
21	8.45	0.84	0.46	0.58	1.94	0.00
22	7.32	0.17	0.38	0.01	2.04	0.00
23	8.19	0.72	0.25	0.02	2.20	0.00
24	6.46	0.30	0.40	0.03	2.34	0.00

Table C-4 Concentration of ammonia, nitrite and nitrate in C:N=8:1 with the glucose as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.00	0.00	0.87	0.00
1	0.01	0.00	0.00	0.00	1.14	0.00
2	5.31	0.09	0.01	0.00	0.84	0.00
3	6.16	0.02	0.03	0.00	0.86	0.00
4	14.93	0.64	0.04	0.00	0.94	0.00
5	20.41	0.68	0.08	0.00	0.85	0.00
6	19.73	1.55	0.10	0.00	1.12	0.00
7	17.40	1.40	0.14	0.00	1.08	0.00
8	14.34	0.28	0.19	0.01	1.29	0.00
9	11.17	0.10	0.17	0.01	1.26	0.00
10	10.80	0.91	0.21	0.01	1.25	0.00
11	10.27	0.39	0.18	0.01	1.26	0.00
12	11.69	0.39	0.11	0.03	1.37	0.00
13	10.10	0.13	0.21	0.01	1.44	0.00
14	9.69	0.43	0.08	0.00	1.46	0.00
15	10.86	0.38	0.08	0.00	1.53	0.00
16	9.98	0.26	0.10	0.00	1.05	0.00
17	11.16	0.24	0.04	0.01	1.87	0.00
18	7.09	0.09	0.02	0.00	2.19	0.00
19	12.31	0.63	0.02	0.00	1.51	0.00
20	6.32	0.04	0.01	0.00	1.69	0.00
21	6.91	0.01	0.01	0.00	2.02	0.00
22	7.06	0.32	0.02	0.00	1.67	0.00
23	5.64	0.17	0.01	0.00	1.87	0.00
24	5.74	0.03	0.02	0.00	1.97	0.00

107

Table C-5 Concentration of ammonia, nitrite and nitrate in C:N=16:1 with the glucose as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.01	0.00	0.00	0.00	0.89	0.00
1	0.00	0.00	0.00	0.00	0.86	0.00
2	5.57	0.27	0.01	0.00	0.81	0.00
3	4.69	0.22	0.02	0.00	0.89	0.00
4	9.85	0.04	0.03	0.00	0.88	0.00
5	13.67	1.32	0.05	0.00	0.90	0.00
6	14.57	0.55	0.06	0.00	1.14	0.00
7	11.42	0.13	0.08	0.00	1.09	0.00
8	8.22	0.11	0.10	0.01	1.26	0.00
9	4.58	0.19	0.08	0.01	1.22	0.00
10	4.31	0.15	0.05	0.01	1.21	0.00
11	2.98	0.06	0.08	0.05	1.26	0.00
12	4.85	0.10	0.01	0.01	1.41	0.00
13	3.33	0.04	0.01	0.01	1.49	0.00
14	4.29	0.03	0.04	0.00	1.59	0.00
15	6.27	0.16	0.10	0.00	1.97	0.00
16	6.87	0.02	0.09	0.00	0.97	0.00
17	6.20	0.22	0.02	0.00	2.03	0.00
18	9.18	0.32	0.03	0.01	2.33	0.00
19	9.33	0.03	0.03	0.00	1.74	0.00
20	8.54	0.64	0.02	0.00	1.92	0.00
21	19.51	2.31	0.02	0.00	2.05	0.00
22	3.12	0.07	0.04	0.00	2.04	0.00
23	3.77	0.08	0.03	0.00	2.18	0.00
24	2.54	0.08	0.04	0.00	2.25	0.00

Table C-6 Concentration of ammonia, nitrite and nitrate in NH$_4$Cl only with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.08	0.00	0.02	0.00	1.06	0.00
1	0.16	0.02	0.03	0.00	1.42	0.00
2	1.31	0.03	0.04	0.00	1.19	0.00
3	2.01	0.12	0.04	0.00	1.15	0.00
4	3.13	0.15	0.06	0.01	1.14	0.00
5	4.00	0.16	0.05	0.00	1.07	0.00
6	3.86	0.08	0.07	0.00	1.04	0.00
7	5.91	0.10	0.07	0.00	1.23	0.00
8	5.67	0.08	0.08	0.00	1.52	0.00
9	7.24	0.26	0.09	0.00	1.18	0.00
10	4.87	0.35	0.11	0.00	1.29	0.00
13	4.93	0.02	0.26	0.02	1.34	0.00
15	5.08	0.17	0.35	0.06	1.48	0.00
18	7.55	0.69	0.42	0.01	1.73	0.00

Table C-7 Concentration of ammonia, nitrite and nitrate in C:N= 2:1 with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.09	0.00	0.02	0.00	0.99	0.00
1	0.12	0.00	0.03	0.00	1.22	0.00
2	1.07	0.02	0.04	0.00	1.17	0.00
3	1.76	0.03	0.04	0.00	1.16	0.00
4	3.06	0.07	0.06	0.00	1.11	0.00
5	3.72	0.17	0.05	0.00	1.51	0.00
6	3.57	0.07	0.06	0.00	1.12	0.00
7	5.26	0.18	0.06	0.00	1.07	0.00
8	4.97	0.03	0.07	0.00	1.03	0.00
9	6.41	0.09	0.07	0.00	1.42	0.00
10	4.47	0.62	0.07	0.01	1.15	0.00
13	4.40	0.11	0.10	0.00	1.19	0.00
15	4.66	0.06	0.11	0.00	1.10	0.00
18	6.55	0.37	0.13	0.01	1.20	0.00

Table C-8 Concentration of ammonia, nitrite and nitrate in C:N= 16:1 with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.05	0.01	0.02	0.00	2.14	0.00
1	0.11	0.02	0.03	0.00	1.12	0.00
2	0.77	0.01	0.04	0.00	0.99	0.00
3	1.23	0.02	0.05	0.00	1.00	0.00
4	0.18	0.05	0.07	0.00	1.24	0.00
5	0.33	0.02	0.03	0.00	1.14	0.00
6	0.10	0.01	0.12	0.01	1.43	0.00
7	0.70	0.01	0.02	0.00	1.02	0.00
8	0.44	0.01	0.02	0.00	1.06	0.00
9	0.44	0.01	0.00	0.00	1.03	0.00
10	0.47	0.01	0.01	0.00	1.41	0.00
13	1.72	0.25	0.02	0.00	1.47	0.00
15	1.21	0.03	0.02	0.00	1.25	0.00
18	1.88	0.05	0.03	0.01	1.37	0.00

Table C-9 Quantity of total suspended solids in NH_4Cl only with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	Total suspended solids (mg/L)	
	Average	SD
2	20.07	12.40
3	14.56	4.94
5	36.65	23.67
7	29.65	12.88
10	11.17	14.38
13	11.43	14.85
15	6.69	3.35
18	10.66	7.22

Table C-10 Quantity of total suspended solids in C:N=2:1 with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	Total suspended solids (mg/L)	
	Average	SD
2	20.59	7.82
3	12.01	4.04
5	23.37	14.45
7	30.79	5.23
10	19.84	8.37
13	10.25	6.17
15	12.23	3.42
18	9.97	6.66

Table C-11 Quantity of total suspended solids in C:N=16:1 with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	Total suspended solids (mg/L)	
	Average	SD
2	34.13	6.81
3	14.14	5.50
5	48.54	5.12
7	114.38	78.01
10	102.21	57.63
13	174.96	28.70
15	119.02	142.80
18	125.29	105.62

Table C-12 Concentration of Chlorophyll-a, b and c in NH_4Cl only with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
6	73.66	55.37	79.21	69.83	219.08	227.60
8	48.68	15.65	28.96	21.34	140.20	40.71
11	75.10	21.88	86.89	12.60	270.42	89.71
13	95.84	27.60	119.89	36.80	357.41	95.19
16	13.33	10.89	13.99	9.96	45.62	38.09
21	158.93	19.12	29.83	15.64	77.77	56.30

Table C-13 Concentration of Chlorophyll-a, b and c in C:N=2:1 with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
6	25.19	13.67	17.13	9.36	44.41	23.46
8	50.49	7.77	29.18	2.44	134.02	3.83
11	58.37	4.43	65.73	5.35	208.51	7.45
13	98.10	19.63	110.81	19.89	345.80	31.30
16	31.61	15.13	30.11	17.62	88.95	53.51
21	158.93	171.03	22.50	15.38	85.16	79.37

Table C-14 Concentration of Chlorophyll-a, b and c in C:N=16:1 with the glucose as carbon addition and using water pond in Chulalongkorn University.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
6	71.91	26.41	44.69	7.09	88.58	47.28
8	122.84	52.73	45.67	18.27	209.12	124.75
11	189.59	44.54	203.01	94.62	555.09	242.44
13	208.70	103.02	196.00	84.88	627.40	232.62
16	122.98	97.40	160.00	226.74	236.03	357.80
21	265.41	69.40	51.29	25.98	152.63	46.80

Table C-15 Concentration of ammonia, nitrite and nitrate in Control without carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
1	0.09	0.02	0.00	0.00	1.25	0.00
2	0.02	0.01	0.04	0.03	1.39	0.00
3	0.08	0.01	0.07	0.08	1.39	0.00
4	0.11	0.02	0.01	0.01	1.37	0.00
5	0.15	0.02	0.02	0.00	1.57	0.00
7	0.04	0.02	0.00	0.00	0.99	0.00
9	0.02	0.04	0.00	0.00	1.25	0.00
11	0.02	0.01	0.15	0.07	1.06	0.00

Table C-16 Concentration of ammonia, nitrite and nitrate in C:N=16:1 with the glucose as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
1	0.01	0.01	0.01	0.03	0.85	0.00
2	0.07	0.12	0.01	0.01	0.91	0.00
3	0.01	0.00	0.00	0.00	0.76	0.00
4	0.01	0.00	0.01	0.00	0.82	0.00
5	0.09	0.02	0.01	0.01	1.04	0.00
7	0.53	0.47	0.00	0.00	0.69	0.00
9	0.00	0.01	0.00	0.00	1.00	0.00
11	0.14	0.18	0.15	0.06	0.99	0.00

Table C-17 Concentration of ammonia, nitrite and nitrate in C:N=16:1 with the starch as carbon addition and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
1	0.04	0.01	0.00	0.00	1.16	0.00
2	0.00	0.00	0.07	0.10	0.98	0.00
3	0.15	0.04	0.01	0.01	1.02	0.00
4	0.01	0.01	0.00	0.00	1.22	0.00
5	0.02	0.00	0.04	0.05	1.28	0.00
7	0.03	0.05	0.00	0.00	0.83	0.00
9	0.02	0.03	0.00	0.00	1.19	0.00
11	0.00	0.00	0.12	0.07	0.95	0.00

Table C-18 Physical parameters measure from Control without carbon addition and using tap water.

Day	Temperature(^{o}C)		pH	
	Average	SD	Average	SD
1	21.80	0.00	8.72	0.05
3	26.80	0.44	8.79	0.14
5	27.05	0.67	8.99	0.30
7	23.58	0.15	9.10	0.34
9	15.73	0.49	9.37	0.70
11	24.55	0.21	9.53	0.51

Table C-19 Physical parameters measure from C:N=16:1 with the glucose as carbon addition and using tap water.

Day	Temperature(°C)		pH	
	Average	SD	Average	SD
1	21.80	0.00	8.51	0.02
3	27.30	0.00	8.62	0.03
5	27.85	0.64	8.89	0.06
7	8.83	0.42	8.83	0.04
9	8.89	1.27	8.89	0.19
11	9.17	0.14	9.17	0.04

Table C-20 Physical parameters measure from C:N=16:1 with the starch as carbon addition and using tap water.

Day	Temperature(°C)		pH	
	Average	SD	Average	SD
1	21.80	0.00	8.64	0.16
3	27.15	0.64	8.45	0.09
5	27.95	0.21	8.83	0.01
7	23.80	0.28	8.98	0.06
9	15.35	0.21	8.90	0.12
11	24.80	0.14	9.25	0.02

Table C-21 Concentration of ammonia, nitrite and nitrate in Control that was added with NH_4Cl (80%) and shrimp diets (20%) as nitrogen source only and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.01	0.00	0.02	0.01	2.45	0.85
1	1.08	0.18	0.02	0.00	1.42	0.21
2	1.57	0.43	0.05	0.04	1.23	0.15
3	2.07	0.63	0.03	0.00	1.44	0.19
4	3.13	0.49	0.05	0.01	1.30	0.15
5	4.34	0.18	0.05	0.01	1.29	0.11
7	3.42	0.16	0.05	0.00	1.36	0.09
8	2.71	0.43	0.06	0.01	1.51	0.05
9	2.04	0.57	0.11	0.02	1.06	0.51
10	1.84	0.55	0.10	0.02	1.26	0.08
11	1.36	0.70	0.13	0.03	1.35	0.04
12	0.83	0.80	0.12	0.04	1.28	0.00
13	0.55	0.71	0.08	0.08	1.00	0.24
15	0.01	0.01	0.08	0.12	1.05	0.13
16	0.22	0.35	0.08	0.13	1.21	0.19
17	0.07	0.08	0.15	0.13	1.08	0.28
18	0.05	0.01	0.07	0.10	1.22	0.19
19	0.10	0.11	0.05	0.08	1.08	0.12
21	0.15	0.10	0.05	0.04	1.19	0.08
23	0.06	0.03	0.01	0.00	1.15	0.07
24	0.06	0.06	0.00	0.00	1.12	0.03
26	0.08	0.02	0.00	0.00	1.27	0.11
28	0.02	0.01	0.00	0.00	1.27	0.16
30	0.03	0.02	0.00	0.00	1.20	0.12
31	0.01	0.01	0.00	0.00	1.28	0.16

ศูนย์วิทยทรัพยากร

จุฬาลงกรณ์มหาวิทยาลัย

Table C-22 Concentration of ammonia, nitrite and nitrate in C:N=2:1 that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	TAN (mg-N/L) Average	SD	Nitrite (mg-N/L) Average	SD	Nitrate (mg-N/L) Average	SD
0	0.01	0.01	0.02	0.00	1.96	0.34
1	0.89	0.27	0.02	0.00	1.38	0.12
2	1.55	0.23	0.03	0.01	1.31	0.07
3	1.65	0.68	0.03	0.00	1.20	0.06
4	2.98	0.15	0.04	0.00	1.18	0.07
5	4.47	0.10	0.04	0.00	1.29	0.06
7	3.13	0.50	0.05	0.00	1.28	0.06
8	2.52	0.72	0.06	0.00	1.44	0.10
9	2.75	0.55	0.07	0.01	1.35	0.05
10	2.41	0.58	0.09	0.01	1.22	0.07
11	2.40	0.28	0.12	0.03	1.31	0.09
12	1.91	0.42	0.13	0.05	1.23	0.10
13	1.06	0.05	0.12	0.06	1.13	0.12
15	0.06	0.05	0.14	0.07	1.21	0.06
16	0.49	0.59	0.15	0.09	1.38	0.04
17	0.34	0.31	0.07	0.04	1.40	0.09
18	0.33	0.26	0.11	0.04	1.45	0.25
19	0.27	0.31	0.05	0.04	1.27	0.13
21	0.54	0.60	0.03	0.04	1.37	0.21
23	0.28	0.23	0.02	0.02	1.46	0.20
24	0.15	0.18	0.02	0.03	1.27	0.14
26	0.13	0.07	0.01	0.01	1.41	0.12
28	0.03	0.02	0.00	0.00	1.32	0.04
30	0.04	0.03	0.00	0.00	1.34	0.14
31	0.03	0.02	0.00	0.00	1.39	0.09

Table C-23 Concentration of ammonia, nitrite and nitrate in C:N=16:1 that was added with NH_4Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.01	0.00	0.01	0.00	2.96	0.14
1	1.03	0.13	0.02	0.00	1.62	0.27
2	1.50	0.31	0.03	0.01	1.28	0.20
3	2.28	0.40	0.04	0.01	1.33	0.11
4	3.03	0.53	0.04	0.01	1.13	0.05
5	4.52	0.72	0.04	0.00	1.21	0.08
7	3.15	0.37	0.05	0.01	1.36	0.27
8	3.29	0.60	0.05	0.01	1.41	0.09
9	3.34	0.87	0.07	0.00	1.28	0.04
10	2.36	0.74	0.08	0.00	1.17	0.03
11	1.53	0.59	0.09	0.01	1.37	0.08
12	0.53	0.67	0.09	0.01	1.19	0.03
13	0.12	0.00	0.02	0.02	0.92	0.05
15	0.03	0.03	0.01	0.01	1.28	0.31
16	0.07	0.06	0.02	0.01	1.30	0.12
17	0.06	0.07	0.01	0.01	1.33	0.13
18	0.05	0.05	0.02	0.02	1.48	0.16
19	0.19	0.17	0.01	0.00	1.45	0.22
21	0.20	0.17	0.03	0.03	1.61	0.13
23	0.13	0.08	0.01	0.01	1.70	0.14
24	0.05	0.04	0.00	0.01	1.57	0.08
26	0.08	0.03	0.00	0.00	1.81	0.06
28	0.03	0.01	0.00	0.00	1.78	0.02
30	0.04	0.01	0.00	0.00	1.87	0.06
31	0.02	0.00	0.00	0.00	1.93	0.06

Table C-24 Quantity of total suspended solids in Control that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
4	66.34	14.33
8	42.71	12.81
10	75.13	33.42
12	132.37	42.23
15	191.57	21.76
17	168.57	43.13
19	111.73	98.35
24	127.27	118.10
28	161.32	139.71
31	149.70	134.17

Table C-25 Quantity of total suspended solids in C:N=2:1 that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
4	79.70	27.35
8	58.18	26.85
10	63.10	44.52
12	98.07	7.95
15	258.89	135.27
17	315.06	163.80
19	176.65	154.45
24	238.89	211.04
28	360.01	327.61
31	331.99	297.53

Table C-26 Quantity of total suspended solids in C:N=16:1 that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
4	55.00	11.96
8	53.38	24.47
10	84.85	59.19
12	167.91	87.03
15	358.97	127.46
17	267.47	38.48
19	377.42	105.77
24	532.90	349.10
28	841.14	485.44
31	717.33	333.08

Table C-27 Concentration of Chlorophyll-a, b and c in Control that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
3	0.00	0.00	0.00	0.00	0.00	0.00
7	27.06	46.88	14.92	25.84	117.03	202.70
9	121.19	53.80	17.31	21.09	81.40	24.26
11	138.33	56.66	13.73	42.43	35.48	88.17
16	106.57	75.01	15.00	25.87	35.48	85.97
18	321.67	208.29	244.82	258.44	805.18	772.33
21	651.15	506.77	384.03	562.23	1325.64	2126.35
26	478.28	568.15	277.57	496.38	899.84	1456.93
31	399.24	30.72	144.81	117.27	662.51	596.16

Table C-28 Concentration of Chlorophyll-a, b and c in C:N=2:1 that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
3	21.51	35.58	33.99	42.02	101.32	141.57
7	39.10	45.71	9.31	24.51	7.11	21.29
9	117.32	107.18	55.71	76.22	185.40	207.33
11	98.91	34.57	120.44	145.41	267.05	157.71
16	498.15	601.63	329.21	598.82	927.74	1657.17
18	348.74	259.04	157.59	217.08	496.71	562.65
21	469.46	54.45	118.21	146.67	249.76	535.44
26	1134.00	781.79	764.35	877.98	2314.33	2556.35
31	1208.52	418.29	582.63	305.04	1720.30	452.70

Table C-29 Concentration of Chlorophyll-a, b and c in C:N=16:1 that was added with NH$_4$Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
3	0.00	0.93	4.66	8.07	26.35	45.64
7	5.80	10.05	0.00	3.76	0.00	4.02
9	134.51	71.28	28.67	43.62	150.11	156.23
11	110.28	111.89	20.16	61.90	54.80	128.89
16	166.66	209.16	76.30	165.14	313.05	576.71
18	253.32	309.94	106.44	232.61	397.03	739.26
21	308.85	227.18	70.46	110.17	415.94	606.22
26	396.34	380.42	196.82	377.16	331.97	770.62
31	1047.03	1035.66	811.71	1054.15	2233.12	2557.96

Table C-30 Physical parameters measure in Control that was added with NH_4Cl (80%) and shrimp diets (20%) as nitrogen source and using tap water.

Day	Temperature (^{o} C)		pH		Alkalinity (mg/L CaCO3)	
	Average	SD	Average	SD	Average	SD
0	31.83	0.60	8.53	0.05	126.67	11.55
2	29.70	0.35	8.60	0.06	-	-
3	27.97	0.15	8.60	0.03	-	-
4	26.77	0.23	8.56	0.06	120.00	0.00
7	28.70	0.30	8.58	0.02	-	-
8	30.20	0.44	8.89	0.10	120.00	17.32
9	31.77	0.25	9.01	0.14	-	-
10	30.70	0.26	9.11	0.19	-	-
11	30.00	0.10	9.07	0.16	-	-
12	35.00	0.44	8.93	0.20	-	-
15	29.35	0.35	8.47	0.29	70.00	28.28
16	34.75	1.20	8.82	0.03	-	-
17	32.40	1.27	9.02	0.06	-	-
18	34.85	0.21	9.11	0.16	-	-
19	33.80	0.57	9.09	0.13	-	-
21	34.05	1.20	9.43	0.19	50.00	0.00
24	31.00	0.35	9.31	0.06	-	-
26	37.10	0.26	9.64	0.06	46.67	5.77
28	33.80	0.79	9.65	0.11	-	-
31	34.17	0.35	9.60	0.08	50.00	0.00

Table C-31 Physical parameters measure in C:N=2:1 that was added with NH_4Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	Temperature (^{o} C)		pH		Alkalinity (mg/L CaCO3)	
	Average	SD	Average	SD	Average	SD
0	32.17	0.29	8.54	0.04	120.00	0.00
2	30.10	0.10	8.60	0.05	-	-
3	28.00	0.61	8.64	0.02	-	-
4	27.00	0.17	8.59	0.06	120.00	0.00
7	28.70	0.46	8.60	0.04	-	-
8	30.37	0.15	8.89	0.08	113.33	5.77
9	31.63	0.45	8.96	0.07	-	-
10	30.77	0.76	9.05	0.10	-	-
11	30.07	0.42	9.03	0.09	-	-
12	35.03	0.25	8.91	0.15	-	-
15	29.70	1.41	8.40	0.12	80.00	14.14
16	35.40	0.14	8.86	0.09	-	-
17	32.95	0.64	9.05	0.12	-	-
18	35.25	0.64	9.09	0.21	-	-
19	34.45	0.21	9.10	0.21	-	-
21	35.15	0.07	9.30	0.33	65.00	21.21
24	30.87	0.97	9.29	0.07	-	-
26	37.40	0.78	9.61	0.22	46.67	5.77
28	34.03	0.51	9.68	0.18	-	-
31	34.57	0.15	9.57	0.22	53.33	5.77

Table C-32 Physical parameters measure in C:N=16:1 that was added with NH_4Cl (80%) and shrimp diets (20%) as nitrogen source and starch as carbon source and using tap water.

Day	Temperature (^{o}C)		pH		Alkalinity (mg/L CaCO3)	
	Average	SD	Average	SD	Average	SD
0	32.47	0.06	8.50	0.01	120.00	0.00
2	30.13	0.23	8.57	0.01	-	-
3	28.20	0.00	8.60	0.02	-	-
4	26.87	0.15	8.57	0.03	120.00	0.00
7	28.77	0.15	8.58	0.02	-	-
8	30.47	0.12	8.85	0.04	116.67	5.77
9	31.90	0.26	8.93	0.03	-	-
10	31.03	0.12	8.99	0.04	-	-
11	30.17	0.12	8.95	0.06	-	-
12	35.20	0.36	8.84	0.03	-	-
15	28.63	0.35	8.31	0.09	80.00	0.00
16	35.53	0.25	8.75	0.06	-	-
17	33.10	0.30	8.91	0.04	-	-
18	35.13	0.29	8.90	0.05	-	-
19	33.97	0.42	8.96	0.03	-	-
21	35.23	0.21	9.03	0.16	83.33	20.82
24	31.37	0.35	9.18	0.30	-	-
26	37.40	0.26	9.48	0.39	70.00	17.32
28	33.97	0.35	9.57	0.25	-	-
31	34.63	0.32	9.46	0.28	63.33	5.77

Table C-33 Concentration of ammonia, nitrite and nitrate in Control was added with feed only and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.01	0.00	0.64	0.04
1	0.36	0.06	0.00	0.00	0.73	0.02
2	2.47	0.47	0.02	0.02	0.84	0.01
3	4.60	0.51	0.03	0.01	1.03	0.08
4	5.12	0.36	0.02	0.01	1.19	0.01
5	7.08	1.10	0.02	0.00	1.18	0.05
6	6.64	3.69	0.03	0.02	1.16	0.05
7	8.24	0.45	0.03	0.01	1.23	0.07
8	8.64	2.65	0.08	0.04	4.65	5.48
9	9.64	3.19	0.11	0.04	1.96	0.18
10	11.02	4.30	0.12	0.05	1.63	0.04
11	17.05	6.17	0.16	0.07	1.74	0.15
12	18.99	6.81	0.38	0.22	1.59	0.12
13	18.51	4.82	2.21	1.93	2.08	0.93
14	8.09	5.96	7.26	2.35	2.96	12.00
15	0.41	0.15	26.85	4.20	18.97	15.57
16	0.83	0.50	28.85	3.93	3.31	4.19
17	0.39	0.35	29.34	3.02	1.82	0.15
18	0.13	0.14	33.26	4.18	1.47	0.14
19	0.13	0.15	34.23	2.44	5.16	1.01
20	0.00	0.00	41.45	6.19	0.00	0.00
21	0.03	0.01	47.18	4.80	0.00	0.00
22	0.04	0.03	47.76	3.41	0.00	0.00
23	0.02	0.02	47.99	4.00	0.00	0.00
24	0.03	0.01	52.34	1.86	2.32	9.88
25	0.02	0.01	43.39	2.14	13.90	4.69
26	0.03	0.01	76.48	4.68	0.00	0.00
27	0.01	0.00	70.25	19.73	0.00	0.00
28	0.03	0.03	46.84	40.57	0.00	0.00
29	0.02	0.02	46.73	40.58	0.00	0.00
30	0.02	0.02	44.78	39.27	0.00	0.00
31	0.02	0.02	34.17	29.60	4.32	4.99
32	0.04	0.04	27.41	24.69	5.51	12.62
33	0.03	0.02	12.62	19.20	20.09	25.92
34	0.02	0.02	10.69	16.00	23.70	25.81
35	0.04	0.04	3.39	4.72	27.51	24.19
36	0.04	0.03	11.93	20.64	22.02	21.88
37	0.02	0.02	0.10	0.16	32.96	29.26
38	0.04	0.03	0.01	0.01	26.43	22.95
39	0.08	0.09	0.01	0.01	32.84	29.06
40	0.04	0.03	0.01	0.01	33.88	30.14
41	0.01	0.01	0.01	0.01	34.89	30.54
42	0.02	0.02	0.00	0.00	32.93	29.22
43	0.02	0.02	0.01	0.01	38.58	34.91
44	0.04	0.06	0.01	0.01	23.91	21.12

Table C-34 Concentration of ammonia, nitrite and nitrate in C:N=2:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.01	0.00	0.66	0.04
1	0.36	0.04	0.00	0.00	0.76	0.09
2	2.35	0.39	0.00	0.01	0.87	0.06
3	2.23	0.70	0.03	0.02	1.04	0.05
4	4.46	0.57	0.04	0.02	1.32	0.03
5	5.45	0.87	0.04	0.02	1.28	0.18
6	6.41	1.04	0.06	0.02	1.23	0.17
7	8.56	2.31	0.08	0.05	1.41	0.24
8	7.93	2.11	0.12	0.07	1.84	0.42
9	7.48	0.21	0.16	0.11	2.14	0.56
10	9.72	0.57	0.25	0.15	1.88	0.34
11	12.98	1.45	0.34	0.16	1.95	0.35
12	15.43	1.56	0.64	0.40	2.22	1.07
13	13.78	3.26	1.75	1.78	2.58	0.99
14	9.61	7.13	6.44	4.54	5.31	13.68
15	7.80	6.57	12.67	9.06	1.02	11.51
16	1.31	1.67	22.43	4.50	0.00	0.00
17	0.13	0.03	25.80	4.14	0.00	0.00
18	0.09	0.08	29.78	0.40	2.50	0.36
19	0.08	0.05	30.66	0.28	3.60	0.80
20	0.04	0.01	39.25	0.10	0.00	0.00
21	0.06	0.01	42.20	1.43	0.01	0.45
22	0.03	0.02	44.13	0.56	0.00	0.00
23	0.04	0.02	44.22	0.10	0.00	0.00
24	0.02	0.00	47.35	0.29	0.00	0.00
25	0.01	0.01	42.01	6.05	3.09	3.31
26	0.03	0.00	77.70	6.59	0.00	0.00
27	0.01	0.00	72.36	17.78	0.00	0.00
28	0.04	0.04	48.72	42.40	0.00	0.00
29	0.04	0.03	42.23	36.87	0.00	0.00
30	0.02	0.02	38.26	33.45	0.00	0.00
31	0.02	0.02	39.35	34.08	1.19	1.30
32	0.04	0.04	34.17	34.31	0.58	14.95
33	0.03	0.04	24.97	34.08	12.71	25.02
34	0.02	0.02	19.33	32.37	19.63	29.79
35	0.05	0.06	15.52	25.44	21.24	25.32
36	0.04	0.04	7.61	13.15	33.40	29.93
37	0.03	0.02	0.02	0.02	39.50	34.29
38	0.02	0.02	0.01	0.01	40.21	35.05
39	0.05	0.04	0.01	0.01	41.03	35.83
40	0.09	0.08	0.18	0.22	42.37	37.08
41	0.02	0.02	0.01	0.01	44.90	39.13
42	0.02	0.02	0.00	0.00	42.56	37.28
43	0.02	0.02	0.01	0.01	48.02	41.85
44	0.03	0.02	0.01	0.01	33.37	29.20

Table C-35 Concentration of ammonia, nitrite and nitrate in C:N=16:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	0.00	0.00	0.01	0.01	0.68	0.04
1	0.39	0.07	0.00	0.00	0.71	0.01
2	1.98	0.38	0.01	0.01	0.87	0.06
3	1.94	0.62	0.02	0.02	1.14	0.06
4	0.75	0.63	0.10	0.12	1.61	0.39
5	0.52	0.63	0.09	0.09	1.58	0.50
6	2.22	1.64	0.26	0.36	1.37	0.66
7	1.37	0.89	0.20	0.21	1.82	0.70
8	2.19	1.21	0.22	0.22	1.91	0.70
9	1.82	1.51	0.20	0.20	2.41	0.95
10	3.56	1.28	0.22	0.18	2.10	0.86
11	2.95	2.00	0.16	0.17	2.40	0.97
12	5.57	2.68	0.24	0.20	2.20	0.65
13	4.24	3.28	0.23	0.23	2.54	0.72
14	5.81	2.27	1.36	1.10	0.00	0.00
15	4.81	3.77	0.55	0.63	0.39	0.40
16	4.96	4.03	3.57	1.14	0.00	0.00
17	6.98	2.85	4.83	5.16	0.00	0.00
18	3.93	5.14	11.18	9.78	1.23	3.79
19	3.72	5.21	12.99	14.70	2.12	3.18
20	0.48	0.44	21.37	7.59	2.77	2.47
21	0.02	0.01	24.24	7.50	1.35	3.38
22	0.30	0.03	25.04	7.89	1.30	6.20
23	0.36	0.42	26.87	12.51	0.96	4.24
24	0.05	0.02	31.32	7.11	1.50	2.90
25	0.05	0.02	36.72	4.70	2.21	6.99
26	0.19	0.10	58.12	28.17	0.00	0.00
27	0.03	0.00	57.13	30.42	0.00	0.00
28	0.28	0.12	47.94	16.35	0.00	0.00
29	0.17	0.10	52.26	11.41	0.00	0.00
30	0.03	0.01	36.34	9.62	2.08	7.66
31	0.03	0.02	38.59	11.30	4.60	3.93
32	0.07	0.05	25.84	10.02	12.69	20.78
33	0.26	0.30	26.09	19.28	20.59	29.47
34	0.07	0.10	24.15	20.30	21.48	32.97
35	0.14	0.13	25.41	21.13	18.37	37.18
36	0.06	0.04	18.43	15.85	29.97	31.83
37	0.09	0.06	19.38	17.75	30.53	35.51
38	0.07	0.04	14.63	13.63	34.18	31.86
39	0.09	0.05	4.12	6.80	42.47	15.46
40	0.37	0.20	0.52	0.25	50.99	16.89
41	0.13	0.12	0.31	0.20	55.27	20.24
42	0.03	0.02	0.02	0.01	55.48	17.93
43	0.08	0.08	0.17	0.06	63.90	17.60
44	0.06	0.03	0.02	0.02	47.48	11.37

Table C-36 Quantity of total suspended solids in Control was added with feed only and stocking density of 3 kg/m^3 in 130 Land using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
6	163.83	15.52
8	216.58	33.75
10	291.92	143.62
12	409.80	41.15
14	435.91	107.21
16	493.26	287.42
18	448.00	107.48
20	439.90	144.82
23	346.99	48.78
25	321.23	35.25
27	341.47	27.22
30	636.04	611.94
33	490.09	252.66
35	294.12	8.32
38	198.96	131.11
41	315.35	30.83
43	351.77	56.08

Table C-37 Quantity of total suspended solids in C:N=2:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
6	159.68	24.11
8	211.11	22.34
10	347.09	33.07
12	404.39	45.69
14	467.98	24.09
16	436.75	87.64
18	592.23	100.98
20	599.40	81.65
23	572.14	130.31
25	585.52	146.19
27	527.96	132.19
30	908.01	248.71
33	533.59	154.53
35	502.73	125.08
38	494.19	188.92
41	555.25	158.95
43	558.82	153.90

Table C-38 Quantity of total suspended solids in C:N=16:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
6	336.78	196.29
8	310.00	60.83
10	349.17	74.59
12	504.90	89.21
14	592.14	101.86
16	602.78	18.81
18	679.06	68.07
20	706.67	50.58
23	753.49	76.48
25	786.63	39.67
27	727.23	90.10
30	906.88	159.28
33	656.60	125.04
35	508.85	216.06
38	853.98	151.72
41	650.04	88.62
43	614.42	211.68

Table C-39 Concentration of Chlorophyll-a, b and c in Control was added with feed only and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
7	883.56	941.36	918.18	1223.82	2320.55	3457.85
11	417.47	143.91	181.45	65.37	271.87	103.85
13	1626.29	911.37	1546.34	1116.22	4059.93	3393.55
15	618.46	717.71	548.28	796.10	1399.98	2211.77
19	497.05	56.29	386.14	120.44	989.61	366.75
22	241.12	342.85	112.06	129.20	196.16	300.46
24	438.54	493.58	322.45	431.93	769.05	1197.72
26	436.17	569.75	449.82	610.36	1319.33	1851.11
28	1296.21	174.72	1519.05	230.40	4237.99	840.56
31	432.22	7.07	333.05	155.66	831.50	610.68
34	366.60	246.92	370.08	307.88	816.76	658.40
36	260.33	27.58	179.62	86.67	369.55	270.41
40	964.81	914.20	1118.68	1091.50	3256.45	3210.29
42	961.12	646.27	1001.62	815.49	2682.51	2628.08
44	662.57	353.12	662.03	437.82	1807.46	1285.78

Table C-40 Concentration of Chlorophyll-a, b and c in C:N=2:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
7	2063.65	1323.53	2358.96	1596.59	6846.39	4696.40
11	1719.95	1778.78	1823.08	2149.99	5168.51	6226.46
13	2253.94	2354.11	2490.50	3050.02	6883.92	8690.64
15	254.53	231.73	158.08	221.37	287.03	491.30
19	219.25	79.71	84.79	5.90	3.92	61.62
22	83.81	4.93	24.53	6.49	0.00	8.91
24	233.32	95.70	81.22	53.16	35.30	14.42
26	108.90	47.91	54.01	23.49	7.09	4.64
28	833.77	871.59	958.25	1089.40	2594.94	3037.78
31	646.80	208.88	588.73	211.01	1467.36	602.92
34	350.65	46.63	269.47	54.10	608.25	166.27
36	828.62	437.43	759.78	489.23	1992.43	1273.07
40	612.15	14.86	651.04	19.70	1904.97	11.06
42	863.17	316.09	807.84	336.35	2196.42	835.21
44	829.46	174.91	808.65	221.49	2181.32	578.53

Table C-41 Concentration of Chlorophyll-a, b and c in C:N=16:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
7	255.84	284.68	244.88	305.55	688.58	913.75
11	499.85	382.87	378.44	418.89	902.96	1316.07
13	720.79	507.88	675.16	545.57	1837.42	1585.71
15	239.94	141.26	54.72	40.41	25.62	59.07
19	0.00	200.91	2712.32	3726.76	6453.81	8902.15
22	8.62	8.32	56.87	88.09	10.56	23.13
24	267.51	245.66	190.44	228.40	432.27	631.10
26	57.21	39.93	18.52	26.21	40.77	19.72
28	861.40	1113.42	0.00	398.24	0.00	431.96
31	567.63	585.69	612.65	684.24	1752.97	2060.79
34	188.53	105.35	158.47	63.52	380.62	207.19
36	1025.76	907.25	1158.86	1130.87	3139.47	3166.82
40	262.52	97.97	265.98	104.03	744.28	278.32
42	413.49	49.15	380.86	36.41	1051.12	184.96
44	1089.14	41.67	1141.00	28.10	3397.38	19.40

Table C-42 Physical parameters measure in Control was added with feed only and stocking density of 3 kg/m^3 in 130 L and using tap water.

	Temperature (^{o} C)		pH		Alkalinity (mg/L CaCO3)	
Day	Average	SD	Average	SD	Average	SD
0	28.93	0.15	8.51	0.06	43.33	5.77
1	32.90	1.01	8.32	0.14	-	-
2	33.63	0.25	8.45	0.06	100.00	10.00
3	34.27	0.78	8.37	0.14	-	-
4	33.87	0.78	8.47	0.09	-	-
5	31.87	0.68	8.69	0.12	150.00	0.00
6	30.80	0.90	8.91	0.10	-	-
7	31.57	0.95	8.85	0.14	-	-
8	33.93	0.83	8.85	0.11	-	-
9	35.90	0.17	6.67	0.29	156.67	28.87
11	33.00	0.20	8.00	0.50	-	-
12	34.17	0.15	8.00	0.00	-	-
13	35.57	0.40	7.67	0.29	-	-
14	33.87	0.12	8.00	0.50	120.00	51.96
15	33.97	0.15	7.33	0.29	-	-
16	0.00	0.00	0.00	0.00	-	-
17	31.80	0.00	7.50	0.00	60.00	14.14
18	33.45	0.35	7.50	0.00	-	-
19	33.85	0.21	7.50	0.00	110.00	0.00
20	33.65	0.21	7.25	0.35	-	-
21	31.75	0.35	7.00	0.00	-	-
22	34.00	0.00	7.00	0.00	-	-
23	35.25	0.35	7.00	0.00	70.00	0.00
24	0.00	0.00	0.00	0.00	-	-
25	30.40	0.14	7.50	0.00	-	-
26	30.00	0.00	7.00	0.00	-	-
27	31.10	0.14	7.25	0.35	-	-
28	30.35	0.78	8.63	0.37	80.00	28.28
30	27.10	0.57	8.66	0.21	-	-
32	0.00	0.00	0.00	0.00	-	-
34	0.00	0.00	0.00	0.00	100.00	0.00
35	27.05	0.21	8.68	0.17	-	-
36	31.55	0.07	9.33	0.12	95.00	7.07
37	32.60	0.14	8.95	0.29	-	-
38	28.05	0.35	8.99	0.23	85.00	21.21
41	33.85	0.21	9.64	0.04	110.00	28.28
43	33.85	0.78	9.58	0.10	100.00	14.14

Table C-43 Physical parameters measure in C:N=2:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Temperature (^{o} C) Average	SD	pH Average	SD	Alkalinity (mg/L CaCO3) Average	SD
0	29.03	0.06	8.53	0.02	46.67	5.77
1	33.27	0.58	8.41	0.05	-	-
2	33.87	0.06	8.46	0.02	110.00	10.00
3	34.47	0.15	8.38	0.07	-	-
4	34.00	0.10	8.43	0.04	-	-
5	31.80	0.26	8.58	0.10	140.00	10.00
6	31.00	0.62	8.72	0.06	-	-
7	31.67	0.58	8.57	0.24	-	-
8	34.07	0.59	8.65	0.06	-	-
9	35.10	1.39	6.50	0.50	150.00	17.32
11	33.17	0.15	8.17	0.29	-	-
12	34.30	0.26	8.00	0.00	-	-
13	35.87	0.12	7.67	0.29	-	-
14	34.07	0.12	7.83	0.58	136.67	41.63
15	34.03	0.15	8.00	0.00	-	-
16	0.00	0.00	0.00	0.00	-	-
17	31.80	0.00	7.50	0.00	90.00	28.28
18	33.50	0.00	7.75	0.35	-	-
19	34.00	0.00	7.50	0.00	105.00	7.07
20	34.00	0.00	7.50	0.00	-	-
21	32.00	0.00	7.25	0.35	-	-
22	34.00	0.00	7.50	0.00	-	-
23	35.60	0.14	7.00	0.00	75.00	7.07
24	0.00	0.00	0.00	0.00	-	-
25	30.55	0.07	7.50	0.00	-	-
26	30.25	0.35	7.00	0.00	-	-
27	31.40	0.57	7.50	0.00	-	-
28	30.95	0.49	8.31	0.08	85.00	7.07
30	27.40	0.57	8.40	0.01	-	-
32	0.00	0.00	0.00	0.00	-	-
34	0.00	0.00	0.00	0.00	65.00	7.07
35	27.30	0.28	8.47	0.01	-	-
36	31.85	0.21	8.25	0.06	110.00	0.00
37	32.85	0.64	8.08	0.04	-	-
38	28.45	0.35	8.40	0.07	85.00	7.07
41	34.95	0.64	8.74	0.40	100.00	14.14
43	34.45	0.64	8.58	0.26	90.00	0.00

Table C-44 Physical parameters measure in C:N=16:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Temperature (^{o} C)		pH		Alkalinity (mg/L CaCO3)	
	Average	SD	Average	SD	Average	SD
0	28.93	0.15	8.53	0.02	43.33	5.77
1	33.00	0.53	8.44	0.05	0.00	0.00
2	33.23	0.99	8.42	0.07	106.67	11.55
3	34.20	0.60	8.28	0.04	0.00	0.00
4	33.80	0.75	8.27	0.05	0.00	0.00
5	31.87	0.47	8.34	0.04	136.67	5.77
6	31.13	0.55	8.42	0.03	0.00	0.00
7	31.80	0.35	8.38	0.07	0.00	0.00
8	34.17	0.64	8.38	0.09	0.00	0.00
9	35.47	1.10	6.00	0.50	173.33	15.28
11	32.63	0.81	7.67	0.29	0.00	0.00
12	34.17	0.15	8.17	0.29	0.00	0.00
13	35.53	0.64	8.00	0.00	0.00	0.00
14	34.07	0.12	7.67	0.29	150.00	10.00
15	33.67	0.58	8.50	0.00	0.00	0.00
16	0.00	0.00	0.00	0.00	0.00	0.00
17	31.67	0.42	8.00	0.00	213.33	23.09
18	33.20	0.50	8.00	0.00	0.00	0.00
19	33.60	0.53	7.67	0.29	203.33	72.34
20	33.63	0.55	7.67	0.29	0.00	0.00
21	31.67	0.58	7.67	0.58	0.00	0.00
22	33.67	0.59	7.83	0.29	0.00	0.00
23	35.27	0.75	7.83	0.29	186.67	76.38
24	0.00	0.00	0.00	0.00	0.00	0.00
25	30.60	0.36	7.67	0.58	0.00	0.00
26	30.23	0.25	7.83	0.29	0.00	0.00
27	31.50	0.50	7.83	0.29	0.00	0.00
28	31.20	0.40	7.87	0.22	110.00	40.00
30	27.63	0.25	8.05	0.24	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00
34	0.00	0.00	0.00	0.00	83.33	45.09
35	27.37	0.12	8.26	0.31	0.00	0.00
36	31.63	0.40	8.08	0.23	110.00	20.00
37	32.80	0.46	7.68	0.47	0.00	0.00
38	28.43	0.23	8.09	0.25	93.33	23.09
41	34.73	0.46	8.11	0.30	96.67	11.55
43	34.23	0.55	8.08	0.30	80.00	10.00

Table C-45 Change of quantity floc volume index (FVI) in Control, C:N=2:1 and C:N=16:1 was stocking density of 3 kg/m^3 in 130 L and using tap water.

Day	Control Floc volume index (ml)		C:N=2:1 Floc volume index (ml)		C:N=16:1 Floc volume index (ml)	
	Average	SD	Average	SD	Average	SD
9	8.00	0.00	1.70	0.00	18.00	0.00
16	33.00	0.00	42.00	0.00	40.00	0.00
24	26.00	9.90	34.50	19.97	58.67	17.01
32	13.00	1.41	28.50	17.06	72.00	14.00
37	14.50	2.12	25.50	20.66	62.00	10.58
44	16.50	2.12	43.50	25.12	82.33	31.56

Table C-46 Concentration of ammonia, nitrite and nitrate in Control was added with feed only and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	1.14	0.05	0.01	0.00	0.90	0.07
1	0.40	0.03	0.01	0.00	0.73	0.03
2	2.83	0.05	0.01	0.00	1.08	0.05
3	4.27	0.12	0.01	0.00	1.23	0.08
4	4.93	0.01	0.01	0.00	1.78	0.36
5	8.14	0.05	0.01	0.00	1.38	0.10
6	8.54	0.56	0.01	0.00	1.44	0.04
7	11.18	0.95	0.02	0.01	1.42	0.09
8	13.67	2.56	0.02	0.00	1.42	0.08
9	15.99	1.79	0.02	0.00	1.49	0.12
11	20.07	1.09	0.02	0.01	1.38	0.12
12	23.61	1.65	0.04	0.02	1.47	0.15
14	26.16	0.49	0.43	0.33	1.56	0.11
15	24.17	2.03	3.33	2.99	1.09	0.55
17	1.29	1.73	13.10	6.91	20.82	7.05
19	0.26	0.15	17.12	19.29	18.08	19.22
20	0.14	0.04	32.13	3.93	5.57	0.78
22	0.24	0.10	28.00	7.35	8.51	4.23
24	0.14	0.01	35.97	4.41	4.20	2.53
26	0.47	0.50	33.39	4.33	12.83	6.29
27	0.07	0.01	32.69	9.17	14.22	4.68
29	0.03	0.02	38.17	7.10	8.30	0.60
31	0.09	0.09	34.48	7.89	13.84	1.23
33	0.04	0.00	39.38	0.00	8.31	0.00
35	0.02	0.00	33.87	0.00	20.95	0.00
37	0.01	0.00	49.23	0.00	9.51	0.00
39	0.02	0.00	31.74	0.00	29.73	0.00
40	0.00	0.00	11.93	0.00	46.44	0.00
42	0.02	0.00	0.42	0.00	56.05	0.00
43	0.02	0.00	0.01	0.00	54.52	0.00
45	0.06	0.00	0.02	0.00	60.03	0.00
47	0.02	0.00	0.03	0.00	53.23	0.00
48	0.04	0.00	0.02	0.00	61.76	0.00
50	0.11	0.00	0.01	0.00	62.28	0.00
51	0.07	0.00	0.04	0.00	62.14	0.00
53	0.00	0.00	0.03	0.00	65.53	0.00
54	0.03	0.00	0.06	0.00	68.02	0.00
56	0.13	0.00	0.01	0.00	53.55	0.00
57	0.02	0.00	0.07	0.00	60.68	0.00
58	0.02	0.00	0.02	0.00	57.87	0.00
59	0.01	0.00	0.10	0.00	68.27	0.00
60	0.01	0.00	0.16	0.00	66.24	0.00

Table C-47 Concentration of ammonia, nitrite and nitrate in C:N=16:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	TAN (mg-N/L)		Nitrite (mg-N/L)		Nitrate (mg-N/L)	
	Average	SD	Average	SD	Average	SD
0	1.30	0.05	0.01	0.00	0.89	0.03
1	0.36	0.00	0.00	0.00	0.76	0.03
2	2.17	0.01	0.01	0.00	1.06	0.02
3	3.08	0.07	0.02	0.01	1.19	0.09
4	2.70	0.09	0.02	0.00	1.69	0.06
5	2.83	0.06	0.03	0.00	1.67	0.09
6	3.29	0.01	0.04	0.02	1.81	0.28
7	3.39	0.60	0.10	0.07	2.11	0.14
8	3.88	0.59	0.17	0.08	1.57	1.42
9	4.77	0.53	0.24	0.08	2.69	0.22
11	5.32	0.04	0.43	0.05	3.11	0.20
12	5.67	0.98	0.51	0.12	3.60	0.04
14	5.65	0.55	0.55	0.13	3.78	0.31
15	6.25	0.79	0.70	0.27	3.82	0.19
17	3.42	0.13	1.54	1.31	3.37	0.16
19	6.52	1.16	0.92	0.29	5.37	1.63
20	9.47	1.75	1.03	0.60	5.12	0.40
22	11.44	1.55	0.77	0.27	5.68	0.14
24	16.92	0.17	1.42	1.04	6.28	0.50
26	13.80	4.30	6.47	6.38	6.73	0.95
27	9.07	11.13	11.76	11.50	8.32	2.34
29	5.22	5.13	14.69	5.91	7.63	2.56
31	0.31	0.04	27.03	0.08	9.24	4.42
33	0.42	0.28	29.24	0.10	9.31	5.49
35	0.35	0.02	28.87	0.05	23.33	6.27
37	0.90	0.64	49.63	4.34	8.33	4.40
39	0.10	0.01	51.05	8.75	18.03	14.00
40	0.01	0.01	47.88	4.21	23.04	10.72
42	0.34	0.28	18.01	14.48	46.41	13.59
43	0.07	0.01	14.55	19.95	41.98	10.86
45	0.07	0.02	9.69	13.02	62.76	20.65
47	0.15	0.15	1.02	1.34	77.82	6.18
48	0.11	0.05	0.11	0.02	85.94	1.93
50	0.45	0.42	0.23	0.02	87.30	10.31
51	0.16	0.03	0.43	0.05	87.68	7.93
53	0.06	0.00	0.16	0.12	99.89	8.55
54	0.05	0.01	0.13	0.05	115.29	15.19
56	0.64	0.22	0.14	0.02	96.42	9.28
57	0.49	0.60	0.89	0.04	107.29	11.15
58	0.05	0.02	0.17	0.15	102.83	14.68
59	0.54	0.08	0.93	0.11	109.22	13.41
60	0.05	0.01	0.61	0.44	108.65	28.81

Table C-48 Quantity of total suspended solids in Control was added with feed only and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
4	34.81	19.46
6	69.96	3.20
8	48.12	12.22
11	117.95	21.76
15	98.08	75.44
16	158.33	11.79
19	164.31	28.72
22	86.10	36.91
26	75.60	41.94
29	94.16	23.17
33	117.95	0.00
37	108.33	0.00
40	113.89	0.00
43	76.39	0.00
48	90.28	0.00
51	104.17	0.00
54	105.71	0.00
57	111.84	0.00
60	275.68	0.00

Table C-49 Quantity of total suspended solids in C:N=16:1 was added with feed and starch as carbon source and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	Total suspended solids (mg/L)	
	Average	SD
4	51.68	17.26
6	118.99	14.74
8	112.03	5.54
11	157.39	26.77
15	232.81	13.60
16	288.15	7.63
19	226.33	78.48
22	304.89	48.09
26	399.10	19.52
29	450.38	10.63
33	453.33	4.71
37	560.32	176.22
40	693.70	190.74
43	749.85	137.48
48	1031.16	181.45
51	1179.98	0.81
54	1181.24	34.62
57	1055.55	46.23
60	1260.62	158.88

Table C-50 Concentration of Chlorophyll-a, b and c in Control was added with feed only and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
3	107.63	108.91	116.44	134.13	428.47	379.22
5	220.03	259.80	293.72	376.83	887.49	909.81
7	60.72	37.24	63.82	52.77	257.03	139.55
9	49.03	21.01	52.76	28.51	249.47	107.86
12	141.02	15.47	256.29	211.31	817.85	295.44
14	68.83	29.05	106.53	29.93	314.80	87.21
17	50.97	58.87	98.26	69.64	389.88	159.72
20	51.35	5.12	41.90	1.05	104.65	27.16
24	22.25	2.54	238.31	296.25	298.00	382.98
27	32.42	9.59	153.75	132.75	23.56	11.55
31	42.73	17.40	146.29	38.70	42.11	59.56
35	133.14	0.00	209.28	0.00	524.99	0.00
39	78.21	0.00	91.72	0.00	184.37	0.00
42	132.70	0.00	283.67	0.00	288.37	0.00
47	143.02	0.00	111.02	0.00	341.32	0.00
50	91.09	0.00	224.26	0.00	30.22	0.00
53	840.24	0.00	1003.89	0.00	2988.58	0.00
56	1769.08	0.00	2048.12	0.00	6273.23	0.00
58	1915.36	0.00	2317.25	0.00	6286.59	0.00

Table C-51 Concentration of Chlorophyll-a, b and c in C:N=16:1 was added with feeds and starch as carbon source and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	Chlorophyll-a (mg/m^3)		Chlorophyll-b (mg/m^3)		Chlorophyll-c (mg/m^3)	
	Average	SD	Average	SD	Average	SD
3	213.61	215.87	261.06	238.87	811.99	788.99
5	552.97	684.18	675.86	847.95	2040.38	190.00
7	1475.31	1839.72	1812.00	2226.90	5308.64	179.00
9	125.81	22.21	111.94	17.68	654.58	66.99
12	100.19	58.69	140.27	19.57	459.29	151.16
14	92.29	101.54	86.51	122.34	614.48	127.37
17	925.47	1277.22	1108.61	1396.78	3378.58	150.00
20	33.06	10.56	157.96	90.84	35.22	49.81
24	69.36	17.99	436.77	266.37	68.53	96.92
27	492.23	123.06	734.41	44.87	1479.96	263.69
31	406.52	521.11	954.36	81.14	1366.38	1230.05
35	359.02	154.17	2581.21	731.23	3528.90	66.33
39	975.28	3.15	2484.28	28.31	5305.35	1000.39
42	462.62	446.05	1393.10	164.69	2128.64	408.30
47	259.92	179.58	979.80	986.31	600.09	240.58
50	404.94	263.94	1111.43	462.16	2116.04	346.32
53	1884.57	848.14	2329.83	897.02	6502.91	2561.46
56	2916.09	1626.33	3524.54	1868.29	9947.78	5255.75
58	2877.53	74.65	3384.99	132.67	9656.01	452.17

ศูนย์วิทยทรัพยากร

จุฬาลงกรณ์มหาวิทยาลัย

Table C-52 Physical parameters measure in Control was added with feed only and stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	Temperature (^{o}C) Average	SD	pH Average	SD	Alkalinity (mg/L CaCO3) Average	SD
1	31.65	0.35	8.61	0.00	130.00	0.00
2	29.60	0.14	8.73	0.02	-	-
3	30.05	0.21	8.74	0.01	-	-
4	31.95	0.21	8.63	0.05	150.00	14.14
5	30.10	0.14	8.74	0.07	-	-
6	27.95	0.35	8.58	0.02	-	-
7	27.95	0.21	8.60	0.01	-	-
8	29.05	0.21	8.60	0.02	-	-
9	28.60	0.14	8.68	0.01	180.00	0.00
11	28.80	0.28	8.75	0.03	185.00	7.07
12	31.70	0.42	8.75	0.07	-	-
14	32.45	0.07	8.42	0.01	-	-
15	33.05	0.21	8.51	0.08	180.00	14.14
17	32.80	0.85	8.69	0.08	-	-
19	31.40	0.57	8.73	0.11	-	-
20	29.40	0.14	8.67	0.39	55.00	7.07
22	30.15	0.21	8.63	0.04	-	-
24	32.12	0.59	8.63	0.04	75.00	7.07
26	27.40	0.28	8.67	0.03	-	-
27	26.70	0.14	8.57	0.03	-	-
29	27.20	0.14	8.89	0.08	90.00	0.00
31	28.75	0.07	8.94	0.14	-	-
33	29.40	0.00	8.86	0.00	90.00	0.00
35	28.70	0.00	8.71	0.00	-	-
37	31.10	0.00	9.28	0.00	100.00	0.00
39	30.20	0.00	8.87	0.00	-	-
40	28.50	0.00	9.48	0.00	-	-
42	25.50	0.00	9.41	0.00	-	-
43	25.90	0.00	9.53	0.00	-	-
45	27.10	0.00	9.41	0.00	90.00	0.00
47	27.10	0.00	9.27	0.00	100.00	0.00
48	28.50	0.00	9.02	0.00	-	-
50	27.80	0.00	8.50	0.00	-	-
51	27.60	0.00	8.73	0.00	100.00	0.00
53	29.80	0.00	8.85	0.00	100.00	0.00
54	28.50	0.00	8.73	0.00	-	-
56	27.30	0.00	9.07	0.00	-	-
57	27.30	0.00	8.85	0.00	110.00	0.00
58	27.60	0.00	8.87	0.00	-	-
59	27.60	0.00	8.87	0.00	-	-
60	27.60	0.00	8.87	0.00	-	-

Table C-53 Physical parameters measure in C:N=16:1 was added with feeds and starch as carbon source and stocking density of 3 kg/m³ in 500 L and using tap water.

Day	Temperature (°C) Average	SD	pH Average	SD	Alkalinity (mg/L CaCO3) Average	SD
1	31.85	0.21	8.59	0.01	130.00	0.00
2	29.60	0.14	8.63	0.03	-	-
3	29.70	0.14	8.61	0.03	-	-
4	31.50	0.28	8.51	0.05	155.00	7.07
5	29.95	0.07	8.54	0.04	-	-
6	27.70	0.14	8.46	0.02	-	-
7	28.75	0.07	8.49	0.03	-	-
8	29.60	0.00	8.47	0.03	-	-
9	28.85	0.07	8.52	0.04	155.00	7.07
11	28.90	0.00	8.50	0.05	155.00	7.07
12	31.45	0.21	8.50	0.05	-	-
14	33.40	0.14	8.33	0.10	-	-
15	33.30	0.00	8.39	0.01	165.00	7.07
17	31.85	0.07	8.49	0.02	-	-
19	31.35	0.07	8.44	0.04	-	-
20	29.35	0.07	8.27	0.04	175.00	7.07
22	30.35	0.07	8.41	0.01	-	-
24	32.20	0.14	8.46	0.04	215.00	21.21
26	27.90	0.00	8.49	0.07	-	-
27	26.85	0.07	8.42	0.01	-	-
29	27.40	0.14	8.48	0.04	170.00	28.28
31	28.50	0.00	8.52	0.08	-	-
33	29.75	0.07	8.52	0.09	115.00	7.07
35	29.00	0.14	8.33	0.08	-	-
37	31.60	0.14	8.88	0.18	115.00	7.07
39	30.50	0.42	8.49	0.05	-	-
40	28.50	0.14	9.06	0.13	-	-
42	25.90	0.14	8.96	0.11	-	-
43	25.75	0.21	9.06	0.10	-	-
45	27.45	0.21	8.70	0.17	110.00	14.14
47	27.60	0.14	8.68	0.08	100.00	28.28
48	28.55	0.35	8.53	0.08	-	-
50	29.00	0.00	8.11	0.02	-	-
51	28.70	0.28	8.27	0.04	110.00	14.14
53	30.00	0.28	8.37	0.07	100.00	28.28
54	28.65	0.07	8.41	0.06	-	-
56	28.10	0.14	8.78	0.16	-	-
57	28.25	0.07	8.33	0.06	90.00	0.00
58	28.15	0.07	8.38	0.07	-	-
59	28.15	0.07	8.38	0.07	-	-
60	28.15	0.07	8.38	0.07	-	-

Table C-54 Change of quantity floc volume index (FVI) in Control and C:N=16:1 C:N=16:1 stocking density of 3 kg/m^3 in 500 L and using tap water.

Day	Control Floc volume index (ml)		C:N=16:1 Floc volume index (ml)	
	Average	SD	Average	SD
9	0.30	0.00	1.35	0.92
19	12.50	6.36	16.00	5.66
24	2.00	0.00	24.00	11.31
31	2.00	0.00	45.50	7.78
39	2.00	0.00	49.00	52.33
45	9.00	0.00	60.00	28.28
53	22.00	0.00	112.50	17.68
60	16.00	0.00	86.00	5.66
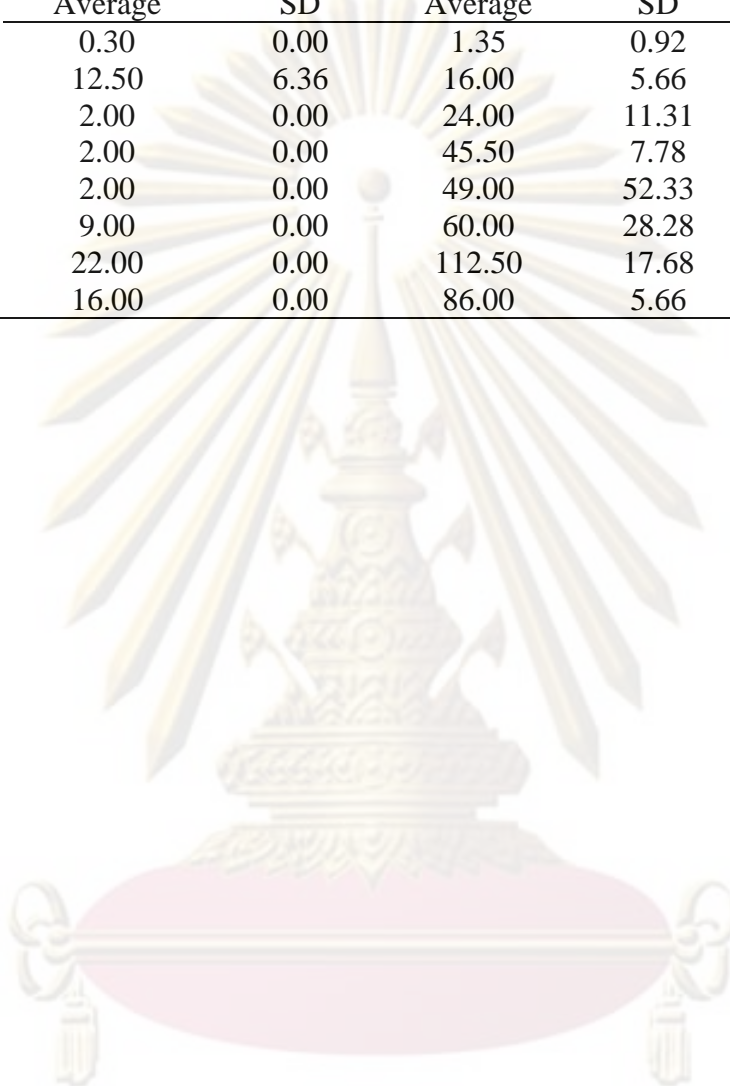
Appendix D

Calculations

D-1 Calculation of Substrate in C:N ratio

1. Nitrogen source (NH₄Cl and Shrimp diets) for biofloc formation

1.1 Ammonium Chloride (NH₄Cl)

Ammonium chloride has molecular weight 53.5 g that was contained nitrogen 14 g. To obtain 1 mg-N/L of nitrogen in 5 L of water, the amount of ammonium chloride required is:

$$\frac{[(0.005\,g - N) \times (53.2\,g)]}{(14\,g)} = 0.0191\,g - N \text{ of ammonium chloride}$$

1.2 Shrimp diets

Shrimp diets contain 35% protein by weight and it is approximated that 1 g of protein contain 0.16 g of nitrogen. To obtain 1 mg-N/L of nitrogen in 5 L of water, the amount of shrimp diets required is:

$$\frac{[(0.001\,g - N/L) \times (5L) \times (6.25)]}{0.35} = 0.0893\,g - N \text{ of shrimp diet}$$

2. Carbon source (glucose and tapioca starch) for biofloc formation

2.1 Glucose

Glucose has molecular weight 180 g that was contained carbon 72 g. The example substrate C:N ratio at 16:1 which added nitrogen with NH₄Cl = 0.0191

g-N. To obtain carbon 0.0191×16 = 0.3056 g-C in 5 L of water, the amount of glucose required is:

$$\frac{[(0.3056\,g - C) \times 180\,g]}{72\,g} = 0.764\,g - C \text{ of glucose}$$

2.2 Tapioca starch

Tapioca starch has carbon approximately 50%. The substrate C:N ratio at 16:1 which added nitrogen with NH_4Cl = 0.0191 g-N. To obtain carbon 0.0191×16= 0.3056 g-C in 5 L of water, the amount of starch required is:

$$\frac{[(0.3056 \times 50) \times 2]}{100} = 0.3056\,g \quad C \text{ of starch}$$

D-2 Calculation of Tilapia growth

Approximately 30% of total fish populations was sampling once every 2 week. The following calculations were used to determine tilapia growth data during the zero-water exchanged cultivations.

$$Average\ length\ (cm/fish) = \frac{Sum\ of\ total\ length}{Number\ of\ fish}$$

$$Average\ weight\ (g/fish) = \frac{Sum\ of\ total\ weight}{Number\ of\ fish}$$

$$Average\ daily\ growth\ (g/day)$$
$$= \frac{Final\ weight\ per\ fish - Beginning\ weight\ per\ fish}{Cultivating\ period}$$

$$Feed\ conversion\ ratio\ (FCR) = \frac{Total\ weight\ of\ feed\ consumed}{Final\ weight - Beginnig\ weight}$$

$$Survival\ rate = \frac{Number\ of\ remaining\ fish\ population \times 100}{Number\ of\ initial\ fish\ stocking}$$

D-3 Calculation nitrogen of Tilapia feeds and nitrogen in Tilapia (modified from Wutikumpoln, 2003)

1. Nitrogen of Tilapia in feeds

Tilapia feeds contains 30% protein. Tilapia was fed at 3% of total fish weight per day. Assuming Tilapia have average weight of 1510.5 g was reared in 500 L tank, the amount nitrogen input from feed can be calculated as:

$$Amount\ of\ nitrogen\ from\ feed = \frac{[(1510.5) \times (0.03) \times (0.3)]}{6.25}$$
$$= 2.175\ g - N/day$$

Concentration of nitrogen from feeds that is delivered per day in 500 L tank is:

$$Concentration\ of\ nitrogen\ from\ feed = \frac{(2.175\ g - N)}{500\ L} \times \frac{(1000\ mg)}{1g}$$
$$= 4.35\ g - N/L$$

2. Carbon source starch which used for substrate C:N ratio

Starch has carbon approximately 50%. The substrate C:N ratio at 16:1 which added nitrogen of tilapia in feeds = 2.175 g-N. To obtain carbon 2.175×16= 34.8 g-C in 500 L of water, the amount of starch required is:

$$\frac{[(2.175\,g - C) \times (50) \times 2]}{100} = 2.175\,g - C\ of\ starch$$

3. Nitrogen in fish

Nitrogen can also be found in protein in Tilapia in biomass. According to Wutikumpoln (2003), tilapia contained approximately 48.87% protein content and nitrogen in protein contained approximately 16%. The dried content of tilapia in biomass was about 1/3 of wet biomass, the amount of nitrogen in fish required is:

$$Nitrogen\ in\ tilapia = \left[\frac{1510.5}{3}\right] \times (48.87\%) \times (16\%) = 39.37\,g - N$$

4. Nitrogen in TSS (Biofloc)

Nitrogen is solids (biofloc) can be found in nitrogen contained in biofloc that can analyze from C, H and N analysis. The nitrogen in biofloc contained approximately 4.24% and total suspended solids in biofloc contained 1260.62 mg TSS/L, the amount of nitrogen in solids required is:

$$Nitrogen\ in\ solids\ floc = (1.260\,g - TSS/L) \times (4.24\%) \times (500L)$$
$$= 26.71\,g - TSS\ in\ 500L$$

BIOGRAPHY

Miss. Worarat Vanitchanai was born on 6 th August, 1984 in Bangkok. Her native home was Samutprakarn province. She finished her secondary school from Ratwinit Bangkeao School in 2003. She got bachelor degree from Chemical Engineering in Faculty of Engineer at King Mongkut's University of Technology Thonburi in 2006. She continued her further study for master's degree in Chemical Engineering at Chulalongkorn University joining the Biochemical Engineering Research Group and achieved completed her Master's degree in October, 2009.

Research publication and presentations

Vanitchanai, W., Powtongsook, S., and Nootong, K., 2008. Effects of organic carbon addition in controlling inorganic nitrogen toxicity for the closed-water aquaculture applications. *In Proc. 34th Congress on Science and Technology of Thailand*, Queen Sirikit National Convention Center, Bangkok, Thailand, October 31-November 2, 2008. (Poster Presentation)

Vanitchanai, W., Powtongsook, S. and Nootong, K., 2009. Organic carbon addition influencing inorganic nitrogen control and population dynamics in outdoor closed-water system. *In Proc. 9th International Phycological Congress*, National Olympic Memorial Youth Center, Tokyo, Japan, August 2-8, 2009. (Poster Presentation)

Vanitchanai, W., Powtongsook, S. and Nootong, K., 2009. Effects of organic carbon addition on inorganic nitrogen control and biofloc characteristics during the closed-water tilapia growout in suspension system. *In Proc. 35th Congress on Science and Technology of Thailand*, The Tide resort (Bangsaen Beach), Chonburi, Thailand, October 15-17, 2009. (Oral Presentation)