รายงานฉบับสมบูรณ์

สภาวะเหมาะสมสำหรับกระบวนการสร้างไบโอฟล็อกเพื่อการเพาะเลี้ยงสัตว์น้ำแบบพัฒนาในระบบหมุนเวียนน้ำแบบปิด

Optimal Condition of Biofloc Formation for Intensive Aquaculture Cultivation in Closed Water Recirculating System

ทุนพัฒนาอาจารย์ใหม่/นักวิจัยใหม่ กองทุนรัชดาภิเษกสมโภช
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บทคัดย่อ

งานวิจัยศึกษาการเลี้ยงสัตว์น้ำในระบบปิดโดยนำแนวคิดของระบบไบโอฟล็อกมาใช้ การวิจัยนี้สามารถแบ่งออกเป็น 2 ส่วนคือ (1) การหาสภาวะเหมาะสมของการเกิดไบโอฟล็อกโดยไม่มีสัตว์น้ำโดยปรับระดับสัดส่วนของคาร์บอนและไนโตรเจนที่เติมลงในน้ำ และ (2) การเพาะเลี้ยงปลานิลแบบปิดในระบบไบโอฟล็อกโดยนำข้อมูลจากในส่วนแรกมาประยุกต์ ผลการทดลองในส่วนแรกพบว่าแป้งมันสามารถทดแทนกลูโคสในการสร้างไบโอฟล็อก และการเพิ่มแร่ธาตุที่จำเป็นจากอาหารกุ้งยังช่วยกระตุ้นการสร้างไบโอฟล็อกให้ดีขึ้น การเติมคาร์บอนและไนโตรเจนที่สัดส่วน C:N เท่ากับ 16:1 โดยน้ำหนัก สามารถควบคุมความเข้มข้นแอมโมเนียและไนไตรท์ได้ดีกว่าระดับ C:N ที่ต่ำกว่า

สำหรับผลการทดลองในส่วนที่สองที่มีการเพาะเลี้ยงปลานิลควบคู่กันพบว่า การเติมแป้งมันและอาหารปลานิลทุกวันลงในถังเพาะเลี้ยงในอัตราส่วน C:N เท่ากับ 16:1 จะสามารถควบคุมปริมาณแอมโมเนียและไนไตรท์ได้ดีกว่าชุดควบคุมที่เติมอาหารปลานิลเพียงอย่างเดียว ปริมาณของแข็งแขวนลอยในชุดควบคุมเพิ่มขึ้นอย่างรวดเร็วจาก 30 เป็น 1,118 mg SS/L ลักษณะของไบโอฟล็อกที่พบในชุดควบคุมและชุดทดลองมีความคล้ายคลึงกัน คือมีรูปร่างที่ไม่แน่นอนและประกอบด้วยสิ่งมีชีวิตหลายประเภท เช่น แบคทีเรียชนิดเส้นใย โรติเฟอร์ หนอนตัวกลม และจุลสาหร่ายในปริมาณเล็กน้อย การวิเคราะห์องค์ประกอบอย่างหยาบของไบโอฟล็อกจากชุดทดลองพบว่ามีปริมาณของคาร์บอนและไนโตรเจนเท่ากับ 34.5% และ 4.2% ของน้ำหนักแห้ง ในขณะที่ในชุดควบคุมมีปริมาณของคาร์บอนและไนโตรเจนเท่ากับ 21.7% และ 2.2% ของน้ำหนักแห้ง ผลสำคัญที่รับจากการทดลองส่วนที่สอง พบว่ากระบวนการนำไนโตรเจนเข้าสู่เซลล์และไนตริฟิเคชั่นมีผลต่อการควบคุมความเข้มข้นของสารอนินทรีย์ไนโตรเจนในระบบไบโอฟล็อก โดยในช่วงแรกก่อนการเกิดไนตริฟิเคชั่นที่สมบูรณ์ การเติมสารอินทรีย์จะกระตุ้นกระบวนการนำไนโตรเจนเข้าสู่เซลล์เพื่อควบคุมความเข้มข้นของแอมโมเนียและไนไตรท์ ในระยะต่อมาเมื่อเกิดไนตริฟิเคชั่นที่สมบูรณ์แล้ว จะพบว่าถึงแม้จะมีการเติมสารอินทรีย์ลงในบ่อเลี้ยงทุกวัน แต่ทั้งกระบวนการนำไนโตรเจนเข้าสู่เซลล์และไนตริฟิเคชั่นต่างล้วนมีบทบาทในการควบคุมความเข้มข้นของสารอนินทรีย์ไนโตรเจนในระบบไบโอฟล็อก โดยไนตริฟิเคชั่นจะมีบทบาทมากกว่า จากผลการทดลองจึงควรมีการศึกษาเพิ่มเติมเพื่อยืนยันผลที่ได้รับและศึกษาถึงความสัมพันธ์ของประชากรแบคทีเรียกลุ่มต่าง ๆในระบบไบโอฟล็อก นอกจากนี้ควรมีการพัฒนาระบบแยกตะกอนที่มีประสิทธิภาพสูง

ABSTRACT

This research focused on the closed-water aquacultures using the concept of biofloc system. The research can be divided into two main sections: (1) determination of optimal condition for biofloc formation without aquacultures by adjusting the carbon and nitrogen mass C:N ratios, and (2) closed-water tilapia cultivation in the biofloc system using the information from the first section. The result from the first section found that tapioca starch could substitute glucose for forming bioflocs and mineral contents in shrimp diet helped stimulating biofloc formation. Addition of carbon and nitrogen sources at the C:N ratio of 16:1 was more capable of controlling TAN and nitrite concentrations relative to using the lower C:N ratios.

The result of the zero-water exchanged tilapia cultivation found that the daily addition of tapioca starch and tilapia feed at the C:N of 16:1 was more effective in controlling TAN and nitrite concentrations than supplying only fish feed. Suspended solids concentration increased rapidly from 30 to 1,118 mg SS/L during the 50 days experiment. Morphologies of biofloc regardless of receiving organic carbon appeared irregular shape with the presence of filamentous bacteria, rotifers, zooplanktons, and microalgae to the lesser extent. Average hydrogen content was similar in both the control and treatment tanks ($\approx$ 4.5% dried weight). Statistical analysis indicated that the carbon and nitrogen contents of bioflocs differed significantly between the control and treatment tanks. Average carbon content of bioflocs in control tanks (21.7 $\pm$ 3.1% dried weight) was approximately 60% less than that in treatment tanks (34.5 $\pm$ 0.9% dried weight) while average nitrogen content of bioflocs in treatment tanks (4.2 $\pm$ 0.4% dried weight) was nearly twice that in control tanks (2.2 $\pm$ 0.3% dried weight). The important outcome obtained from the zero-water exchanged tilapia cultivation was the role of nitrogen assimilation and nitrification in the control of inorganic nitrogen levels in a biofloc aquaculture system. Assimilation and nitrification were important for inorganic nitrogen control at different stages of the experiment. In the first stage, prior to the occurrence of complete nitrification, addition of organic carbon promoted nitrogen assimilation into microbial flocs. In the following stage, nitrification and to a lesser extent assimilation were both responsible for effective inorganic nitrogen control. Based on the results, further study is necessary to identify ecological relationships between nitrifying and heterotrophic bacteria in biofloc systems. In addition, biofloc management strategy, specifically the development of an effective biofloc separator, is necessary to improve the efficiency and sustainability of biofloc aquaculture systems.

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INTRODUCTION

1.1 Motivations

Aquaculture is the cultivation of aquatic animals 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 exporting 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 in cultured units produced during the cultivation can also cause adverse health effects on farmed animals, and the release of production water without proper treatment is able to create 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 is 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 but 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). Accumulation 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 biological treatment systems have been developed to maintain ammonia and nitrite concentrations in culture water. Phytoplankton-based systems are attractive due to their simplicity and low operational cost but fail to sustain a stable operation because of periodic phytoplankton bloom and crash cycles. Nitrifying biofilters have been successfully employed in various aquacultural applications. Despite many advantages, the use of nitrifying biofilters remains costly, and more importantly, does not permit the recycling of unconsumed nitrogen in feed (Avnimelech, 2006). This is particularly important because protein is the most expensive component in feed, yet on average, cultured animals use only 25% to 30% of provided nitrogen (Avnimelech 2006). Currently, biofloc systems have been receiving attention for closed-water shrimp and tilapia cultivation because they feature high production yield, water quality control, and feed protein recycling simultaneously in the same culture unit (Avnimelech 2006; Crab et al. 2007; Little et al. 2008). In such systems, inorganic nitrogen control is based on an enhancement of heterotrophic bacterial growth to assimilate nitrogen into new cellular proteins (Avnimelech 2006, Schneider et al. 2007). As bacteria increase biomass, reaching a high density, they tend to form noticeable aggregates (bioflocs), which can be consumed by some cultured animals as a natural food source (Burford et al. 2004; Schneider et al. 2006). Addition of carbon and nitrogen sources at a high C:N ratio into aquaculture ponds was recommended for the establishment of bioflocs and control of inorganic nitrogen concentration (Avnimelech 1999).

After an extensive literature search, work in biofloc systems in Thailand is extremely limited. Hence, it is appropriate to initiate the research that develops the biofloc system that is inexpensive and suitable for the tropical climate found in Thailand. Therefore, this work intends to determine the optimal conditions for biofloc formation and then applying the obtained data to cultivate aquacultures without any water exchange. In addition, the work intends to examine the effects of organic carbon addition on inorganic nitrogen conversion processes in biofloc system and evaluates the role of assimilation and nitrification. Additional information about biofloc composition is also presented.

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 Assess the role of biological processes that are responsible for the nitrogen control in the biofloc system.

1.3 Scopes

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 and 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 can be grown at high density in aerated-mixed ponds. With increasing aquaculture biomass, water quality becomes a limiting factor due to accumulation of toxic compounds particularly ammonia and nitrite. Three different approaches are used to control water quality including (1) exchanging cultured 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 Evolution of pond intensification, 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

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 Nitrogen in aquaculture pond

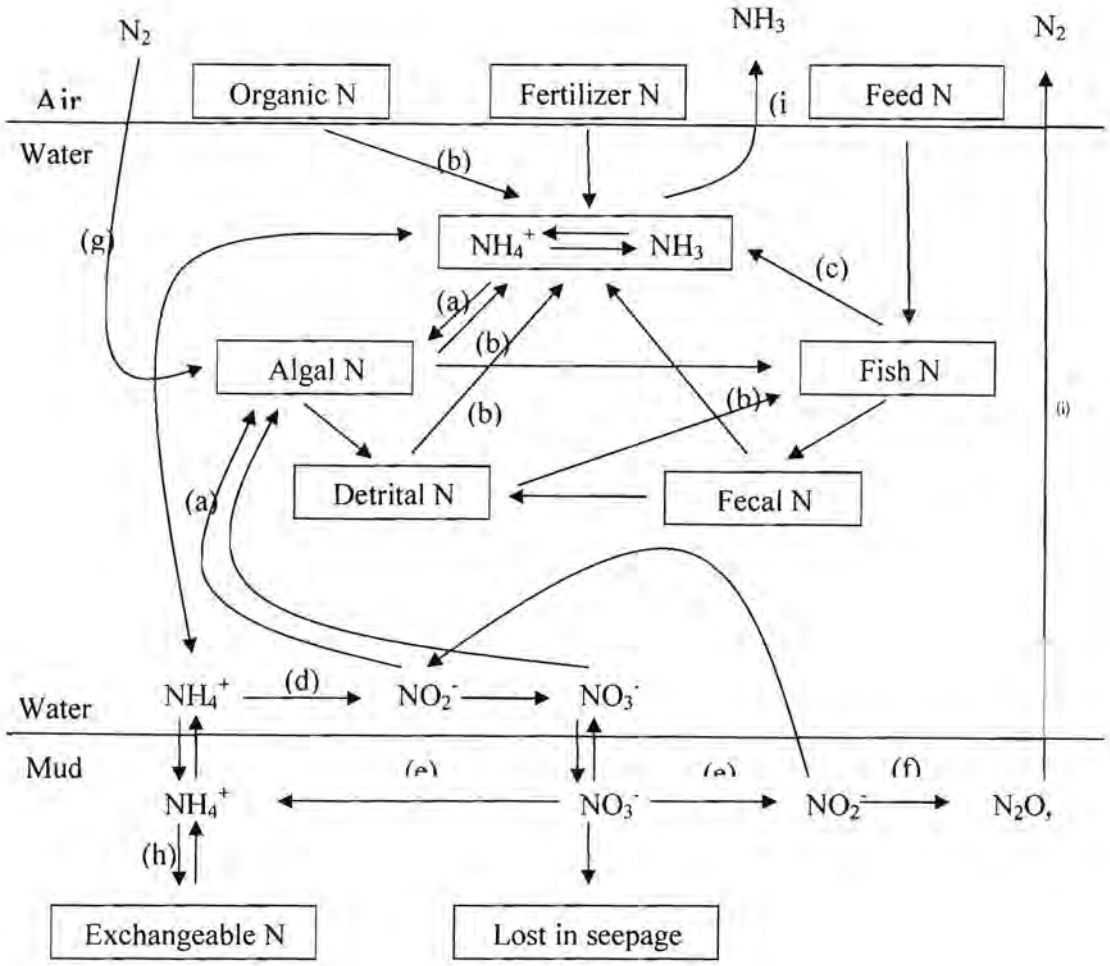


Fig. 2.1 Nitrogen cycle in aquaculture ponds. The illustration is simplified by omitting the food chain between algae and fish. Major processes illustrated are (a) assimilation (b) mineralization (c) excretion (d) nitrification (e) nitrate reduction (f) denitrification (g) biological nitrogen fixation (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 inputs are from various sources such as feeding, biomass decay or aquatic animal excretion; and undergo many biological reactions both in water column and in sediments to change their forms. These biological reactions are essential for natural water treatment in the ponds and are used as the basis for the design of water treatment and recirculating systems for commercial aquacultures.

2.4 Important inorganic nitrogen compounds

Ammonia

Ammonia is introduced into aquaculture ponds via feeds, aquatic animal excretion and the biological degradation of unconsumed feeds. Ammonia is available in water in two forms as NH_3 or NH_4^+ depending on the pH of water. Free ammonia (NH_3) is more toxic towards aquatic animals 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 neurological and cytological failure in fish. The acceptable level of ionized ammonia (NH_4^+) is 1.0 mg N/L.

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 carcinogen. Infant under the age of 6 months may become seriously ill and die, if untreated, after drinking water containing nitrite (Nootong, 2008). For the purpose of aquaculture, it is desirable to keep nitrite concentration under 1.0 mg N/L.

Nitrate

Nitrate is an end-product of nitrification. Nitrate, although is 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., 2003). Discharge of nitrate into

natural water resource quickly accelerates eutrophication by stimulating the growth of microalgae.

2.5 Biological processes for inorganic nitrogen treatment

Treatment of inorganic nitrogen compounds (i.e., ammonia, nitrite and nitrate) is accomplishable 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).

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 were 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. 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 quickly enhances 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).

$$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \rightarrow C_5H_7O_2N + 6.06H_2O + 3.07CO_2 \quad \text{(Bacteria)} \quad (2.1)$$

$$16NH_4^+ + 92CO_2 + HPO_4^{2-} + 92H_2O + 14HCO_3^- \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106O_2 \quad \text{(Microalgae)} \quad (2.2)$$

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 (i.e., 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, 2008). Equation 2.3 and 2.4 represent nitrifying reactions by AOB and NOB.

$$NH^{+}_{4} + 1.5\ O_2 \rightarrow NO_2^{-} + 2H^{+} + H_2O \qquad \text{(AOB)} \qquad (2.3)$$

$$NO^{-}_{2} + 0.5O_2 \rightarrow NO_3^{-} \qquad \text{(NOB)} \qquad (2.4)$$

Many environmental factors affect nitrification. Effective nitrification is observable when the dissolved oxygen (DO) concentration is greater than 2.0 mg/L. Pure cultures of *Nitrosomonas* and *Nitrobacter* exhibited a stoppage of nitrification when the 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 °C, with the optimal temperature at 30 °C (Bitton, 1994). Marine nitrifying bacteria were reported to have optimal temperature range from 30 to 35 °C (Bitton, 1994). The optimal pH for nitrification is reported in the range between 7.0 and 8.5. The presence 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., 2003). 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 (Bitton, 1994).

Heterotrophic Denitrification

Heterotrophic denitrification is a biological process that reduces nitrate into nitrogen gas by denitrifying bacteria under oxygen-limited or anaerobic conditions. Denitrifying bacteria includes many strains namely *Achromobacter, Bacillus denitricans, Flavobacterium, Micrococcus denitrificans, Dinitrobacillus, Spirillum* and *Pseudomonas stutzeri* (US EPA, 1975). Denitrifying bacteria 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. Denitrification increases the pH of water since it generates hydroxyl ion.

$$6NO_3^- + 5CH_3OH \rightarrow 5CO_2 + 3N_2 + 7H_2O + 6OH^- \quad (2.5)$$

Many environmental factors 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 the accepted DO values should not exceed 0.5 mg/L (Christensen and Harremoes, 1977). Denitrification is active under wide temperature range from 0 to 50 °C with the optimal values reported between 35 and 40 °C (Winker, 1984). 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. 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.

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. Anammox does not require organic carbon to produce nitrogen gas (Khin and Annachhatre, 2004 but has the disadvantage compared to heterotrophic denitrification because the bacteria responsible for this process, *Plantomecetales*, have an extremely slow growth with the doubling time about 11 days (Khin and Annachhatre, 2004). 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 except strictly anaerobic. Recently, anammox activity was commonly noticed in sediments.

2.6 Inorganic nitrogen treatment in biofloc systems

Biofloc system features inorganic nitrogen treatment and feed protein utilization in the same cultured unit. Biofloc system is applicable in both freshwater and marine environments (Azim et al., 2008) and is sustainable in both intensive and extensive aquaculture productions (Hari et al., 2006; Avnimelech, 2006; Crab et al., 2007). Research works on bioflocs system have focused mainly on the cultivation of shrimp and tilapia. According to Fig. 2.2, the removal of inorganic nitrogen

compounds (*e.g.*, NH_3, NH_4^+, NO_2^-) in bioflocs technology systems is engineered based on enhancing heterotrophic bacterial growth to assimilate nitrogen into new cellular proteins during microbial biomass synthesis. As bacteria in water flourish reaching high density, they tend to form noticeable amorphous aggregates (*i.e.*, bioflocs) (Burford et al., 2003). Other components in bioflocs include autotrophic bacteria, microalgae, zooplankton, protozoa, inorganic matters (*e.g.*, sand) and the remains of death microorganisms (Avnimelech, 2006). Structure of bioflocs is irregular and highly-opened with the porosity ranged from 65 to 75% of the total aggregate volume. Cruz (1995) described structural similarity between bioflocs in aquaculture systems and those normally found in activated sludge processes.

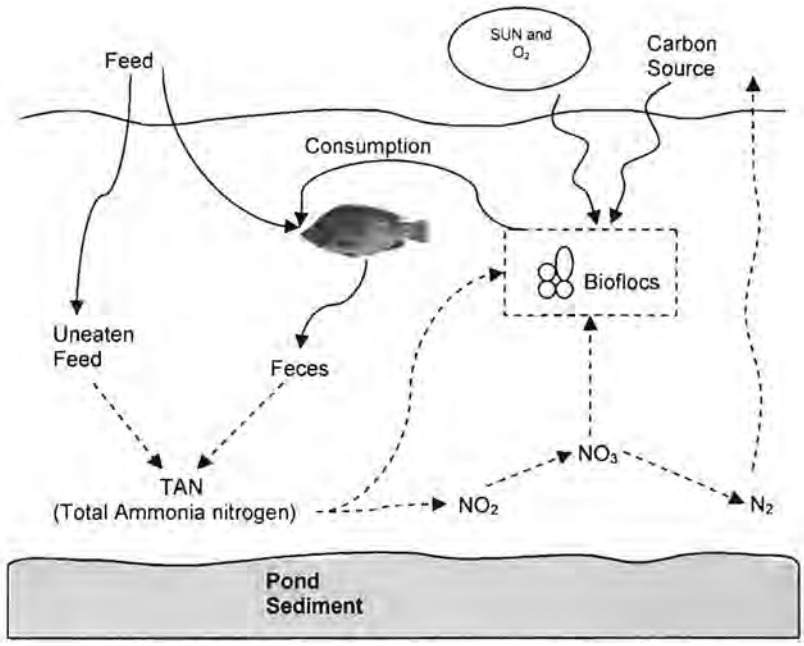


Fig. 2.2 Inorganic nitrogen treatment and protein reutilization concept in a biofloc system (Nootong, 2008)

Engineering of Bioflocs Technology

Equation (2.6) indicates the reaction scheme describing inorganic nitrogen assimilation into new microbial biomass (*i.e.*, $C_5H_7O_2N$). This process, if properly adjusted to enhance microbial nitrogen uptake, could effectively reduce excessive ammonia and nitrite accumulations in water. Clearly, addition of organic compounds (e.g., molasses, starch, cassava meal) into production ponds, heavy aeration and good mixing are important for establishing bioflocs systems.

$$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \rightarrow C_5H_7O_2N + 6.06H_2O + 3.07CO_2 \quad (2.6)$$

1. Addition of organic carbon

Transition of ponds to more heterotrophic dominance could be accomplished and maintained by providing sufficient amounts of organic carbon or other forms of carbohydrate. 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 feeds (Boyd and Clay, 2002). Excessive accumulation of inorganic nitrogen compounds in biofloc systems could be avoided by maintaining high substrate C:N ratios in ponds under aerobic condition. Avnimelech (1999) described a complete removal of 10 mg NH_4^+-N/L within the period of 2 hours following the addition of glucose at the concentration 20 times higher than that of total ammonia nitrogen (TAN). Fontenot et al. (2007) used molasses and ammonium salts to adjust the C:N ratios of shrimp aquaculture wastewater and obtained the result that the optimal C:N ratio was 10:1 for the most effective wastewater treatment. Similar results were reported by Azim et al. (2008) that the optimal bioflocs development measured in term of volatile suspended solids (VSS) and BOD_5 was observed at the C:N ratio of 11.6.

2. Aeration and Mixing

Constant aeration and good mixing of water are essential for maintenance of biofloc system. Bioflocs ponds could be considered as the 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/L (Boyd and Clay, 2002). Malfunction of aeration equipment in biofloc systems could lead to a rapid decrease of oxygen inventory. The availability of dissolved oxygen in water may determine bioflocs structures. Filamentous bacteria tends to dominate when DO is

less than 2 mg/L whereas larger and more compact bacteria bioflocs are more abundant at higher DO levels (i.e., DO = 2.0 – 5.0 mg/L) (Martin et al., 2004). Schryver et al. (2008) recommended operating bioflocs ponds at high DO level (i.e., flocs volume index (FVI) > 200 mL/g) to avoid bioflocs settling too quickly into dead zone region of ponds.

Feed Reutilization in Biofloc Systems

An important feature of bioflocs system, 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 bioflocs consumption by aquacultures. Aquatic animals in biofloc systems are able to consume proteins in feeds at least twice; once in feed and later from bioflocs proteins. Aquaculture systems employing bioflocs 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. (1994) 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. For saline cultivating systems, nitrogen recovery rates were comparable to those in freshwater. Nitrogen recovery rates in a super-intensive shrimp cultivation in Belize were roughly 39% of the quantity available in feeds (Boyd and Clay, 2002). Nitrogen recovery rate was almost double the values typically reported in traditional shrimp cultivation with frequent water exchange (Boyd and Clay, 2002). Additional work focusing on extensive shrimp farming by Hari et al. (2004) utilized biofloc concept by adding 0.39 kg of tapioca flour as supplemental carbon source per kg of 25% protein shrimp diets during *Penaeus monodon* production. Result 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.

Disadvantages

Biofloc system has several disadvantages. Due to high heterotrophic growth, significant amounts of suspended solids are produced during aquaculture production causing high turbidity of water 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. Therefore, a weekly sludge draining must be performed as means 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 (Boyd and Clay, 2002). Alkalinity in forms of $CaCO_3$ and $NaHCO_3$ is required to maintain optimal pH and alkalinity ranging between 7 and 8 and between 100 and 150 mg/L $CaCO_3$, respectively. Another limitation of bioflocs system is high oxygen consumption and mixing requirement to ensure that heterotrophic bacteria are suspended in water and able to degrade added carbon aerobically. Oxygen demand in bioflocs 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.

CHAPTER III

METHODOLOGY

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

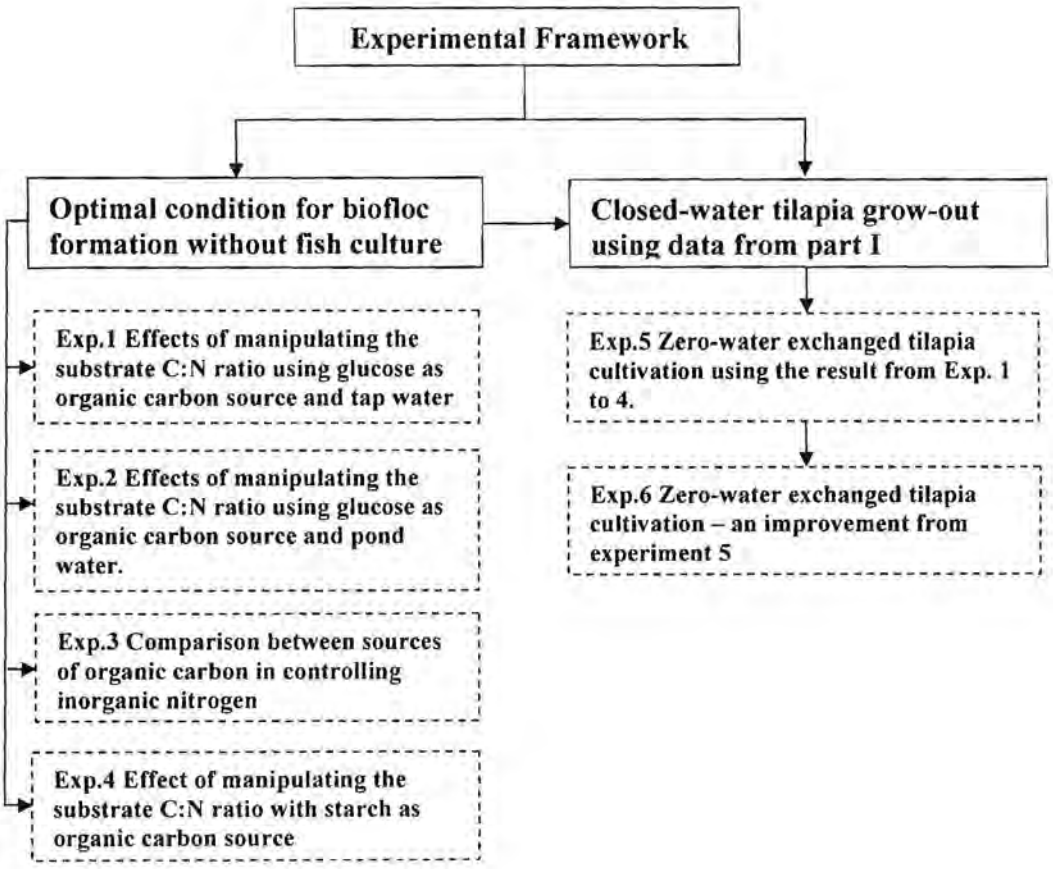


Fig. 3.1 Framework of the experiments in this study

3.1 Optimal condition for biofloc formation without aquacultures

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

The experiment was conducted in a control (n = 3) and 4 treatments (n = 3) 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 the control and treatments to provide different substrate C:N ratios. The control and treatment were 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 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, respectively. One diffusive stone aerator was placed in each glass bottle to provide adequate mixing and dissolved oxygen (DO) at greater than 3.0 mg/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by the 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. Daily water samples 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).

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

The experiment was conducted in a control (n = 3) and 2 treatments (n = 3) by using 9 identical glass bottles (7 L), which were filled up with natural water from pond located near the Faculty of Science at Chulalongkorn University, to attain the working volume of 4 L. Glucose and ammonium chloride were used as the carbon and nitrogen sources, respectively. Different amounts of glucose and ammonium chloride were added daily into the control and treatments to provide different sets of substrate C:N ratios. The control and treatments were 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 and 160 mg were added into the treatments on the daily basis to produce the substrate C:N ratios of 2:1 and 16:1, respectively. One diffusive stone aerator was placed in each glass bottle to provide adequate mixing and DO at greater than 3.0 mg/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by the 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. Daily water samples

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).

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

The experiment was conducted in a control (n = 3) and two treatments (n = 3) 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 (ETC International, Bangkok, Thailand) were used as carbon source while ammonium chloride remained as the nitrogen source. The control and treatments was supplied daily with 30.6 mg NH_4Cl only to attain the ammonium dose at 2.0 mg N/L. Treatment 1 was also supplied daily with 320 mg of glucose to achieve the C:N ratio of 16:1. Similarly, treatment 2 was added with 512 mg of starch to produce the C:N ratio of 16:1. One diffusive stone aerator was placed 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 the 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 water samples were obtained from each glass bottle and immediately analyzed for TAN, nitrite and nitrate according to APHA (1998).

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 (n = 3) and two treatments (n = 3) 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 (ETC International, Bangkok, Thailand) 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 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 was supplied on a daily basis with 15.3 mg NH_4Cl, 17.7 g of 20% shrimp diets and 66 mg of starch to achieve the substrate C:N ratio of 2:1. Treatment 2 was provided daily with 15.3 mg NH_4Cl, 17.7 g of 20% shrimp diets and 528 mg of tapioca starch to attain the substrate C:N ratio of 16:1. A diffusive stone aerator was placed in each glass bottle to provide adequate mixing and DO at greater than 3.0 mg/L. The pH and alkalinity were maintained between 7 and 8 and between 100 and 150 mg/L, respectively by the 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 water samples were obtained from each glass bottle and immediately analyzed for TAN, nitrite and nitrate according to APHA (1998).

3.2 Zero-water exchanged tilapia cultivation in a biofloc system using the result from section 3.1.

3.2.1 Experiment 5: zero-water exchanged tilapia cultivation in a biofloc system

Zero-water exchanged tilapia cultivation was carried out in a biofloc system using the result from section 3.1. Six circular plastic tanks (upper inner diameter of 105 cm; lower inner diameter of 86 cm; height 78 cm) were filled with freshwater from ponds at the Chulalongkorn University to attain 500-L working volume. The initial total suspended solids concentration in the pond water was 25 mg SS/L. Mixing and circulation were provided with a submersible pump and three air diffusers that were placed in each tank to maintain dissolved oxygen (DO) concentrations greater than 4.0 mg/L. Each tank was covered with a semi-transparent lid to prevent the uncontrolled addition of rainwater.

Male Nile tilapia, *Oreochromis niloticus*, from Manit Farm (Phetchaburi Province, Thailand) with an average weight of 30 ± 5 g were stocked in each tank to obtain an initial biomass of approximately 3.0 kg/m^3 (≈ 45 to 47 fish per tank). Tilapia were grown without intentional water exchange for 50 days, and were fed twice daily at 0900 and 1700 with 35% protein commercial feed pellets (Manit Farm Brand, Thailand) at 3% of total fish weight per day. Control tanks (n = 3) were supplied on a daily basis with feed only while treatment tanks (n = 3) were supplied daily with feed plus tapioca starch (ETC International Trading, Bangkok, Thailand) at a weight C:N ratio of 16:1, relative to the nitrogen content of feed input. The proximate weight composition of tapioca starch was: starch 85%, moisture 12.5% – 13.0%, ash 0.2%, and pulp 0.2 cm^3 (ETC International Trading, Thailand). Calculation of starch requirement was based on fish density and used the following assumptions: feed contains 35% protein; feed is provided at 3% of total fish weight per day; protein is 16% nitrogen; approximately 75% of total nitrogen in feed ends up in water; and 50% of starch dry matter is carbon. The pH was maintained between 7 and 8 and alkalinity was maintained between 100 and 150 mg/L $CaCO_3$ by periodic addition of $NaHCO_3$. Tilapia grow-out was conducted without any solids removal. Water samples from each tank were obtained daily at 1400 and immediately analyzed for total ammonia nitrogen (TAN), nitrite, nitrate (NO_3-N) and total suspended solids according to APHA (1998). Prior to sampling, water in each tank was manually agitated using a long wooden stick (length ≈ 2 m) to re-suspend settled solids. Biofloc (i.e., settleable solids) volume was determined after 30-min sedimentation in an Imhoff cone (APHA, 1998). Water samples from the tanks in each group were

combined and settled in an Imhoff cone, and the settled solids dried overnight for analysis of carbon, hydrogen, and nitrogen contents using a CHN analyzer (Perkin Elmer PE2004) based on the Pregl-Dumas method. Statistical analysis (t-test: paired two-sample for means) for TAN, nitrite, nitrate, and suspended solids concentrations between the controls and treatments were calculated using Microsoft Excel 2007.

CHAPTER IV

RESULTS

4.1 Laboratory experiment to determine the optimal condition for biofloc formation

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

Experiment 1 was conducted to obtain the preliminary data for biofloc formation. Since tap water had low amount of microorganisms, approximately 5 g of sediment from nitrifying biofilters treating water from shrimp cultivating tank in the same laboratory were used as initial microbial seeding. Glucose was chosen as an initial carbon source because it was easy to obtain commercially and more importantly it was easily consumed by microorganisms. The source of nitrogen is ammonium chloride. The daily addition of ammonium chloride at 1.0 mg N/L resulted in the accumulations of TAN followed by nitrite while the extent of inorganic nitrogen accumulations was clearly related to the amount of glucose added daily (Fig. 4.1). TAN concentrations were higher than the acceptable limit (i.e., 1.0 mg N/L) for practical aquacultures (Fig. 4.1A). TAN concentrations in the control and treatments increased from negligible levels (i.e., TAN < 0.1 mg N/L) to reach the maximum values ranged from 14.6 to 23.4 mg N/L before declining after day 6. The addition of glucose and ammonium chloride in the treatment receiving the C:N ratio of 16:1 was able to maintain lower TAN concentrations than the control and other remaining treatments. Nitrite concentrations were significantly lower than practical aquaculture limit (i.e., 1.0 mg N/L) for most of the experiment (Fig. 4.1B). The maximum nitrite concentrations ranged from 0.96 to 1.75 mg N/L were observed in the control and treatments after TAN concentration started to diminish on day 6. The daily supplement of glucose and ammonium chloride in the treatments receiving the C:N ratio of 8:1 and 16:1 was capable of producing the average nitrite concentration below 0.2 mg N/L for the entire experiment. Nitrate concentrations were relatively lower than TAN and nitrate concentrations and appeared to increase gradually for all treatments to suggest the occurrence of nitrification (Fig. 4.1C). The highest nitrate concentration was observed in the control, which was supplied on the daily basis with ammonium chloride only. Lower nitrate concentrations were associated with the treatments receiving glucose addition. Moreover, water in the treatment receiving the C:N ratio of 16:1 was the most turbid compared to other treatments due to proliferation of phytoplankton.

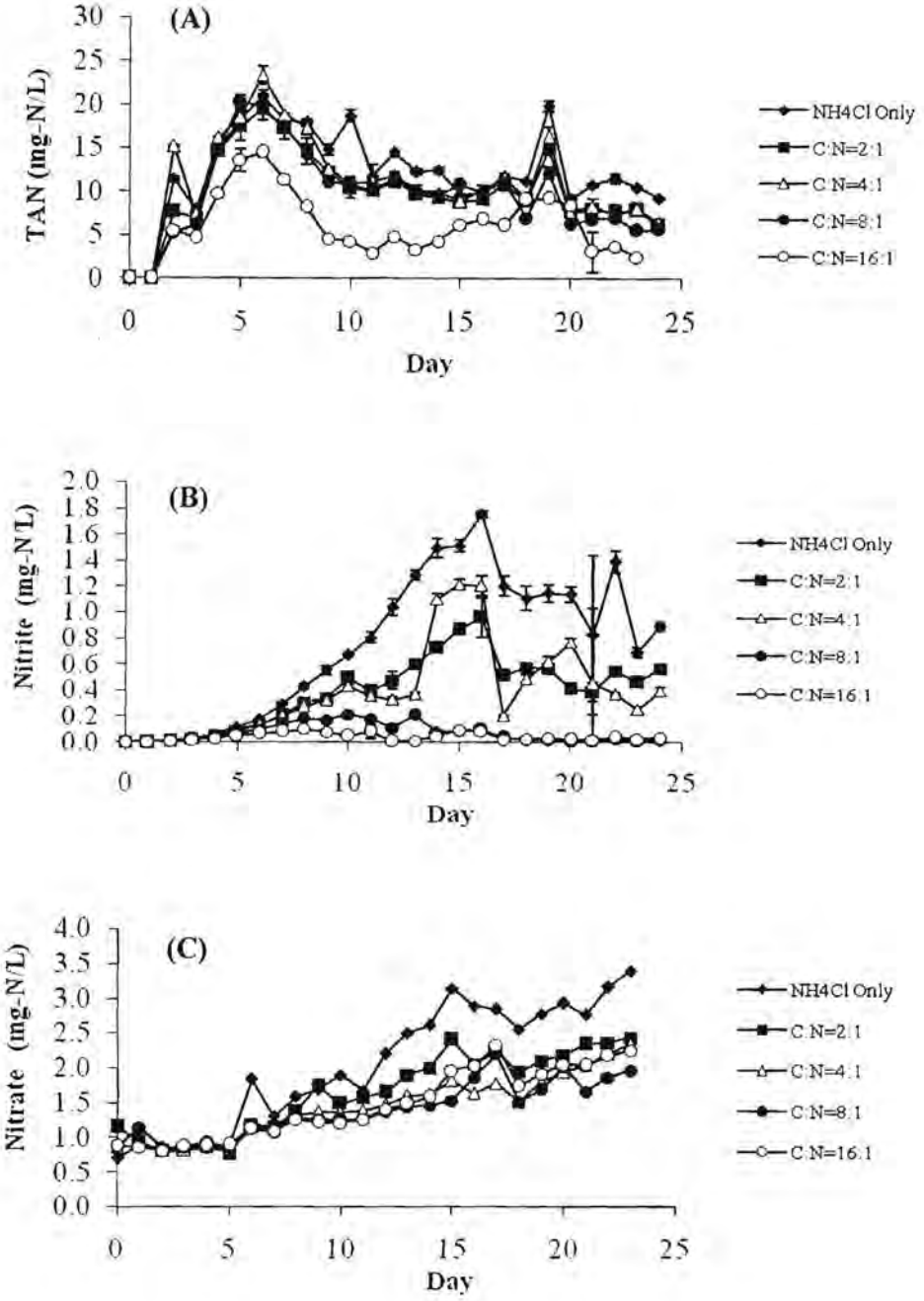


Fig. 4.1 Effect of changing the substrate C:N ratio on the concentrations of TAN (A), nitrite (B), and nitrate (C). The control and treatments received different amounts of glucose and ammonium chloride as followed: (Control) NH_4Cl only, (Treatment 1) C:N = 2:1, (Treatment 2) C:N = 4:1, (Treatment 3) C:N = 8:1 and (Treatment 4) C:N = 16:1.

4.1.2 Experiment 2: preliminary data and the effects of manipulating the substrate C:N ratio using glucose as organic carbon source and pond water

In this experiment, natural pond water near the faculty of Science at the Chulalongkorn University was used to provide microbial seeding. Once again, the daily addition of glucose and ammonium chloride were the carbon and nitrogen sources for bacteria, respectively. An initial suspended solids concentration of pond water was at 25 ± 5 mg SS/L. TAN concentrations were higher than the acceptable limit of 1.0 mg N/L in the control and treatment 1 for most of the experiment (Fig. 4.2A). In those systems, TAN concentrations increased from less than 0.5 mg N/L to reach the maximum concentration measured at 8.0 mg N/L on day 9 before starting to decline. Treatment 2 receiving the C:N ratio of 16:1 was more effective in maintaining TAN concentrations compared to other treatments, showing the TAN concentrations less than 2.0 mg N/L for the entire experiment (Fig. 4.2A). Nitrite concentrations in the control receiving zero glucose addition increased more rapidly compared to other treatments but the nitrite concentrations was still below the acceptable level (Fig. 4.2B). Glucose addition appeared more effective in controlling nitrite with the average nitrite concentrations less than 0.2 mg N/L. Nitrate concentrations were in the range from 1.0 to 1.5 mg N/L for all treatments and did not seem to be affected by varying amount of glucose addition (Fig. 4.2C). During the experiment, the color of water changed due to phytoplankton growth (Fig. 4.3). For the control and treatment 1, phytoplankton flocculated at the end of experiment, resulting in a clear separation from water. The reason of this observation was still unknown at this stage. Measurement of chlorophyll concentrations in the control and treatment 1 showed a slight increase from 20 to 180 mg/m^3, suggesting slow phytoplankton growth (Fig 4.4). A more rapid phytoplankton growth occurred in treatment 2 receiving the C:N ratio of 16:1 as the water changed from light green to dark shading in less than a week. Chlorophyll concentrations in treatment 2 increased from 15 to 265 mg/m^3, a significant difference from the control and treatment 1. Suspended solids concentrations also followed the trend of algal bloom, increasing from 34 to 250 mg SS/L (Fig. 4.4). Microscopic examination of biofloc samples from treatment 2 receiving the daily C:N ratio addition of 16:1 demonstrated that biofloc morphology was irregular and contained ranges of microorganisms including phytoplankton, filamentous bacteria, rotifers, protozoa and detritus. Clearly, 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 control. Applying high substrate C:N ratio at 16:1 was still effective in maintaining acceptable TAN and nitrite concentrations in comparison to using lower substrate C:N ratios.

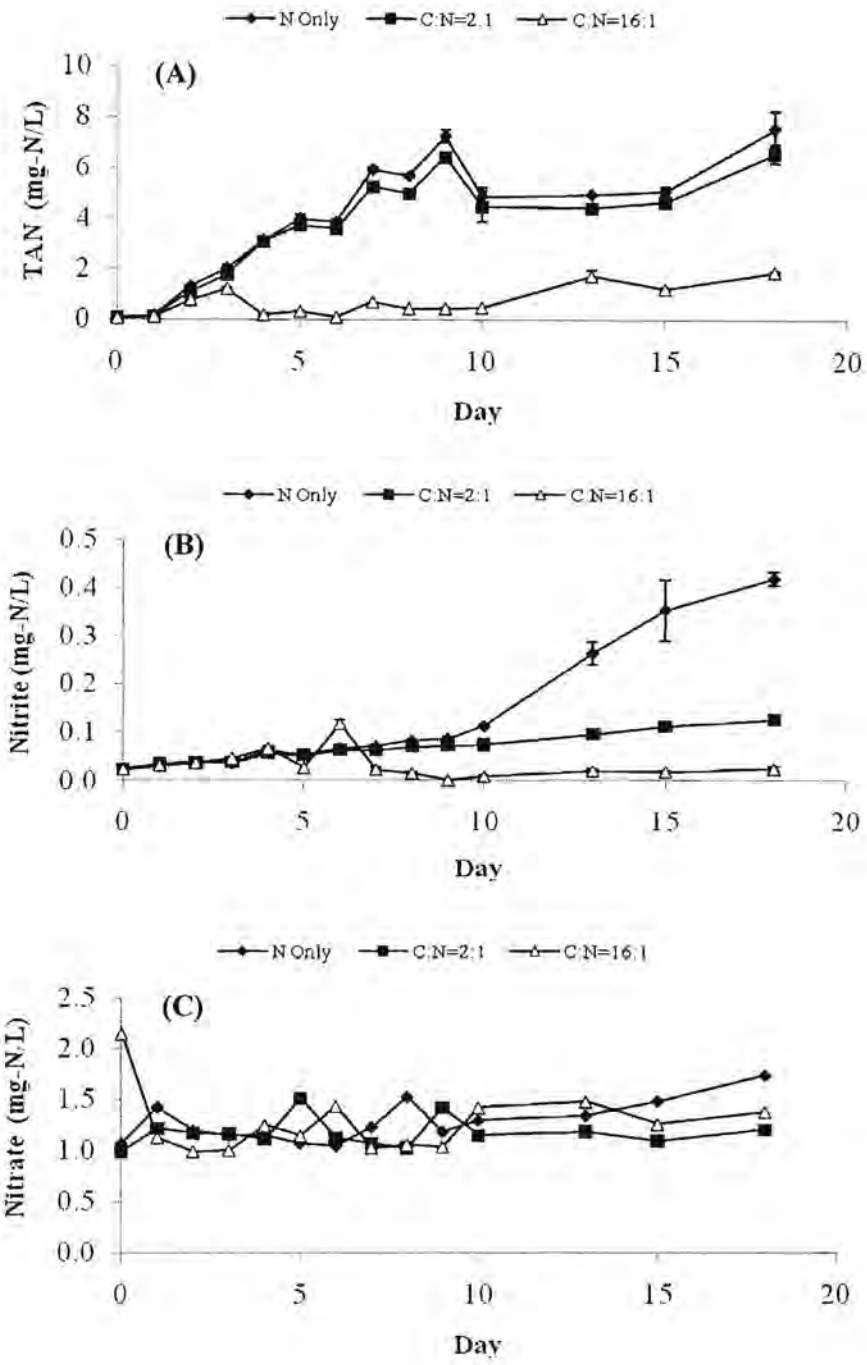


Fig. 4.2 Concentration profiles of TAN (A), nitrite (B) and nitrate (C) in experiment 2 that used pond water to provide initial bioflocs. Tapioca starch and ammonium chloride were added daily into each glass bottles as followed; (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.

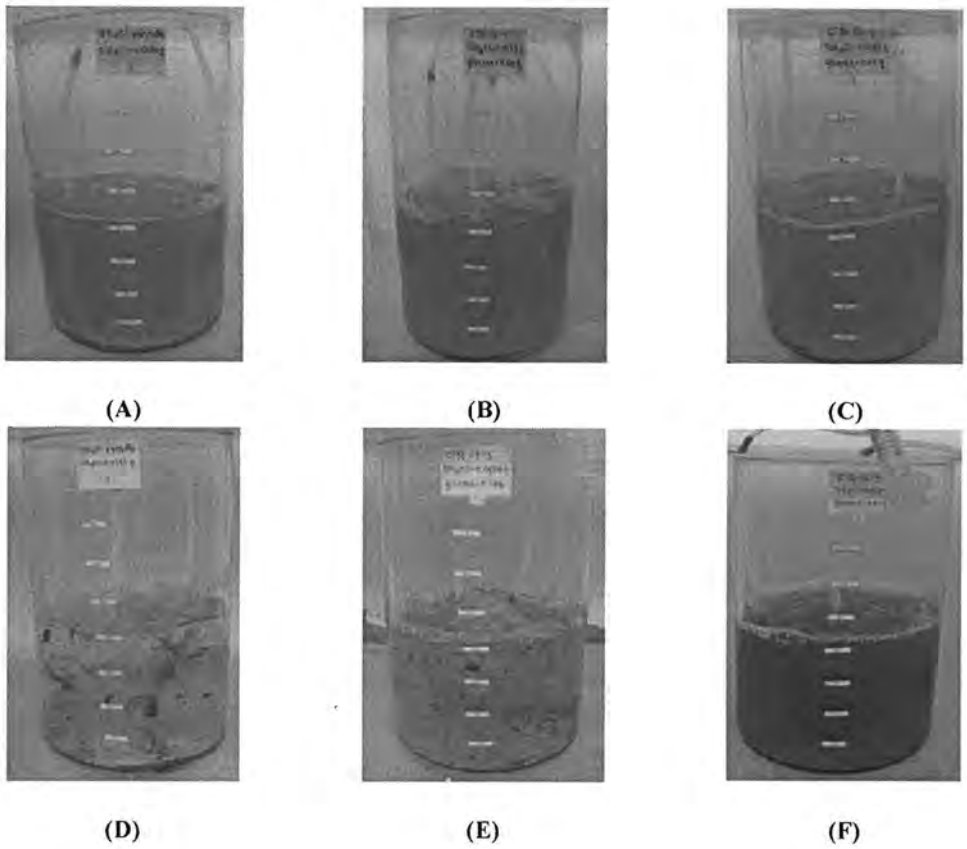


Fig. 4.3 Water characteristics after the addition of tapioca starch and ammonium chloride at different C:N ratios: (A) control on day 1 (B) treatment 1 on day 1 (C) treatment 2 on day 1 (D) control on day 18 (E) treatment 1 on day 18 (F) treatment 2 on day 18.

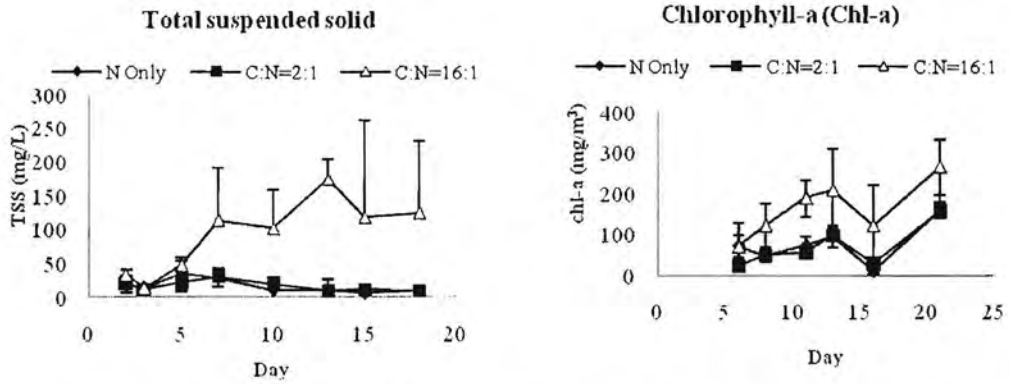


Fig. 4.4 Suspended solids and chlorophyll concentrations in experiment 2 that used pond water to provide initial bioflocs: (Control) nitrogen only, (Treatment 1) C:N = 2:1 and (Treatment 2) C:N = 16:1.

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

This experiment was conducted to compare the effect of using two different organic carbon compounds, glucose and tapioca starch, in controlling inorganic nitrogen concentrations. Tapioca starch was expected to be more practical than glucose in the actual aquacultures because it is available in large quantity and cheaper than glucose. Since the purpose of this experiment was comparing the effectiveness between glucose and tapioca starch, the tap water was used. Ammonium chloride, once again, was the nitrogen source and was supplied daily into each treatment at 2.0 mg N/L while the C:N ratio was maintained at 16:1. TAN and nitrite concentrations were less than 1.0 mg N/L regardless of organic carbon sources while nitrate concentrations were between 0.8 and 1.6 mg N/L (Fig. 4.5). The average TAN concentrations were 0.106 ± 0.177 and 0.034 ± 0.048 mg N/L for treatment 1 (glucose) and treatment 2 (starch), respectively. The average nitrite concentrations from both treatments were similar measured at 0.024 ± 0.053 mg N/L for glucose and 0.030 ± 0.043 mg N/L for starch. The statistical analysis (t-test) indicated that TAN concentrations were insignificantly different between treatments receiving glucose and tapioca starch (Table 4.1). Similar result was obtained for nitrite concentrations. Therefore, glucose can be substituted by tapioca starch as the carbon source to maintain acceptable inorganic nitrogen concentrations 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 ($P < 0.05$)

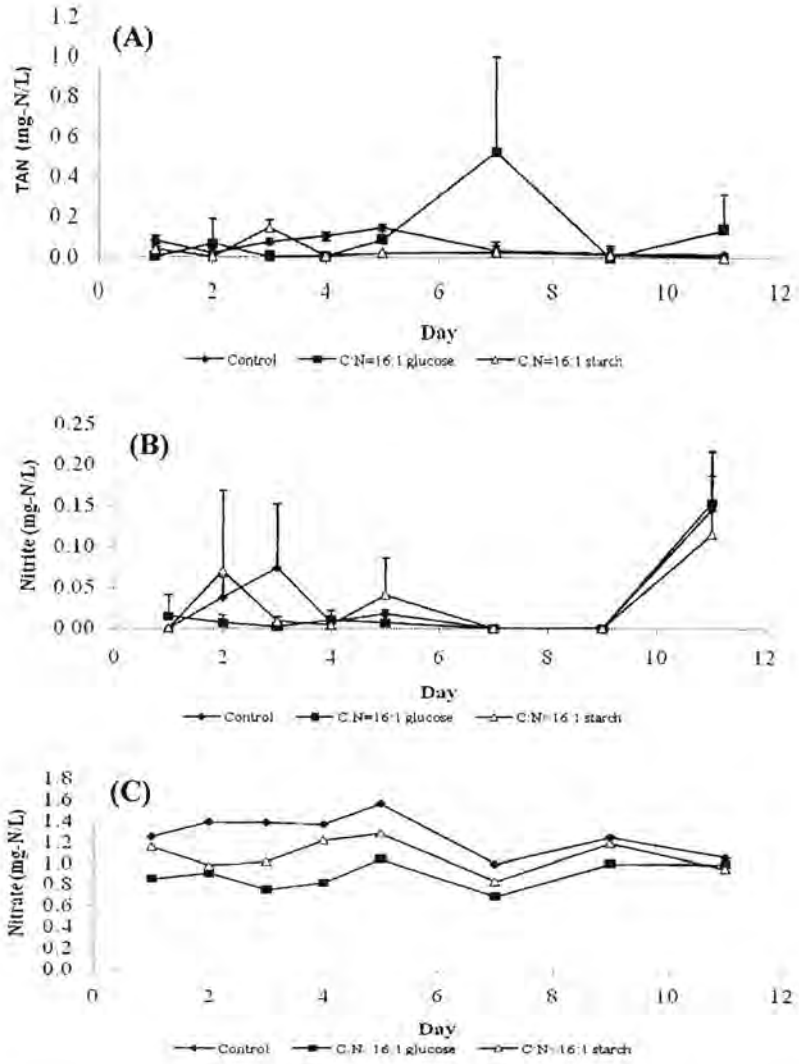
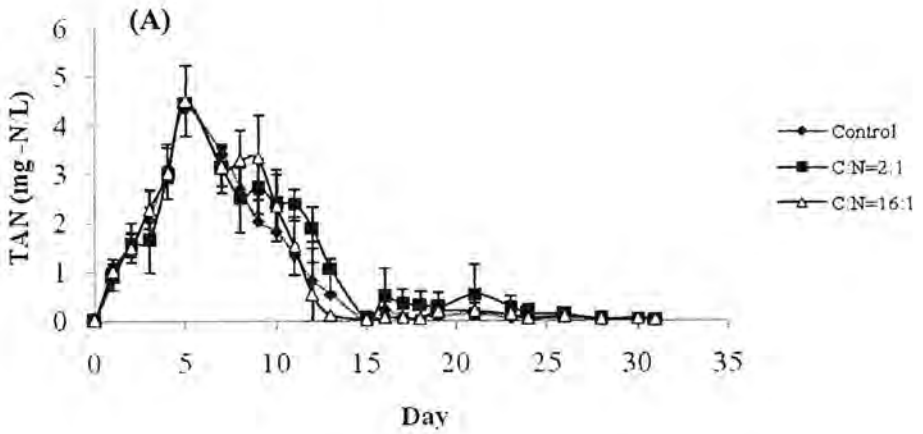


Fig. 4.5 Inorganic nitrogen concentrations from the control and treatments: (A) control with daily addition of nitrogen only, (B) Treatment 1 with daily addition of glucose at C:N = 16:1 and (C) Treatment 2 with daily addition of tapioca starch at C:N = 16:1.

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

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 combined and added daily into the control and treatments at 1.0 mg N/L. Shrimp feed was used as an additional nitrogen source

in this experiment because it contained essential trace elements and metals required for bacterial growth. No significant difference was observed for TAN concentrations in each treatment (Fig 4.6A). Maximum TAN concentrations, approximately 5.0 mg N/L in each treatment, were reached on day 5 before rapidly decreasing below 1.0 mg N/L by day 13. Nitrite concentrations accumulated since the first day of experiment and started to diminish after day 15, with the concentrations less than the acceptable limit (i.e., 1.0 mg N/L) throughout the experiment (Fig. 4.6B). Treatment 2 receiving the C:N ratio of 16:1 was slightly more effective in controlling nitrite than the control and treatment 1 as can be demonstrated by a more rapid decrease in nitrite concentrations between day 10 and day 20. Insignificant difference in nitrate concentrations was observed between the control and treatments where nitrate concentrations ranged from 1.0 to 2.0 mg N/L (Fig. 4.6C). The levels of suspended solids formation were clearly affected by varying the magnitude of C:N ratio addition (Fig 4.7). Suspended solids concentrations in treatment 2 receiving the C:N ratio of 16:1 increased significantly from less than 25 to 800 mg SS/L, whereas substantially lower suspended solids concentrations were observed in the control and treatment 1. Suspended solids concentrations in treatment 1 were also slightly higher than the control. Chlorophyll concentrations increased during the experiment but it was the chlorophyll-c that accounted for the highest amount at the end of the study (Fig 4.7). The concentrations of chlorophyll-a, chlorophyll-b and chlorophyll-c in treatment 2 were 1047, 812 and 2233 mg/m^3, respectively by the end of experiment. The presence of chlorophyll-c suggested that diatoms developed into the dominant phytoplankton species in water. Factors influencing the population dynamics of phytoplankton needed to be further investigated in the future. Table 4.2 summarizes water quality data measured during experiment 4.



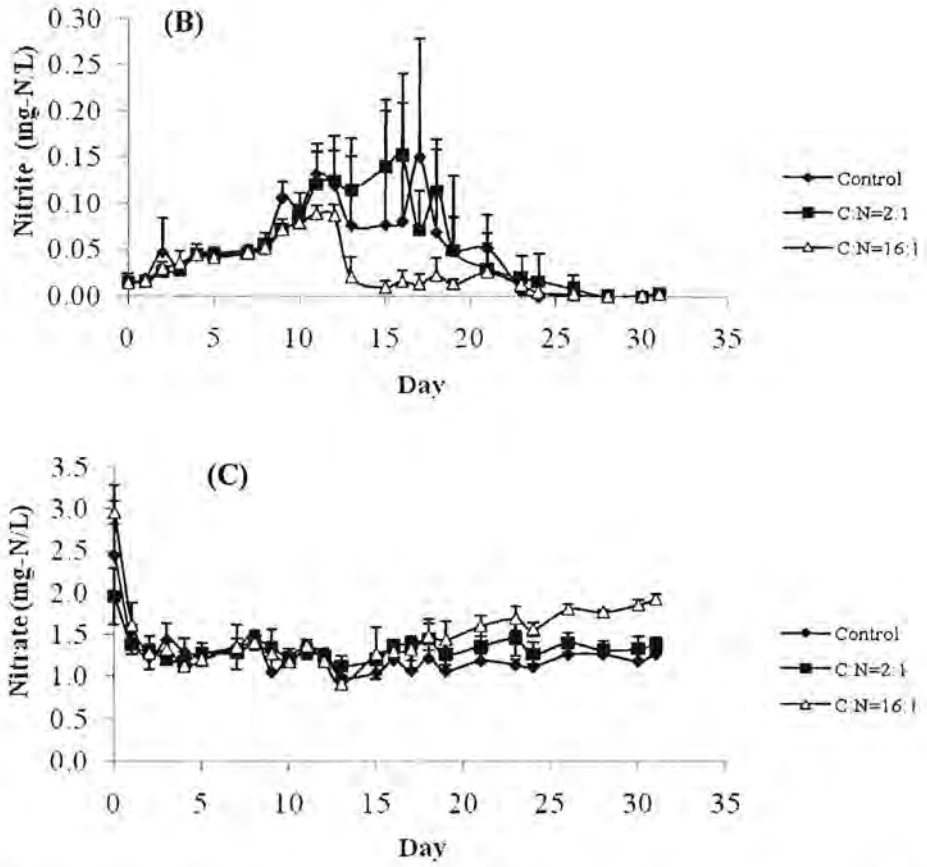
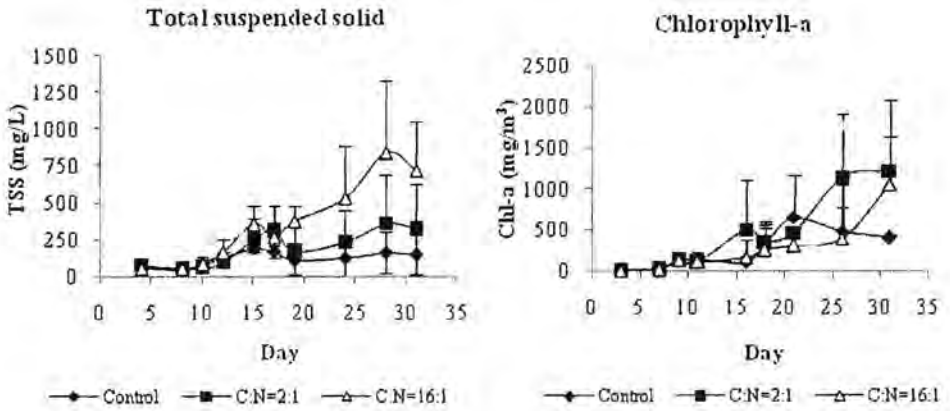


Fig. 4.6 Inorganic nitrogen concentrations in control and treatments with tapioca starch as a sole carbon source and NH_4Cl/shrimp diets as combined nitrogen sources: (A) nitrogen sources were added to the control daily, (B) C:N ratio of 2:1 was added daily to treatment 1 and (C) C:N ratio of 16:1 was added daily to treatment 2.



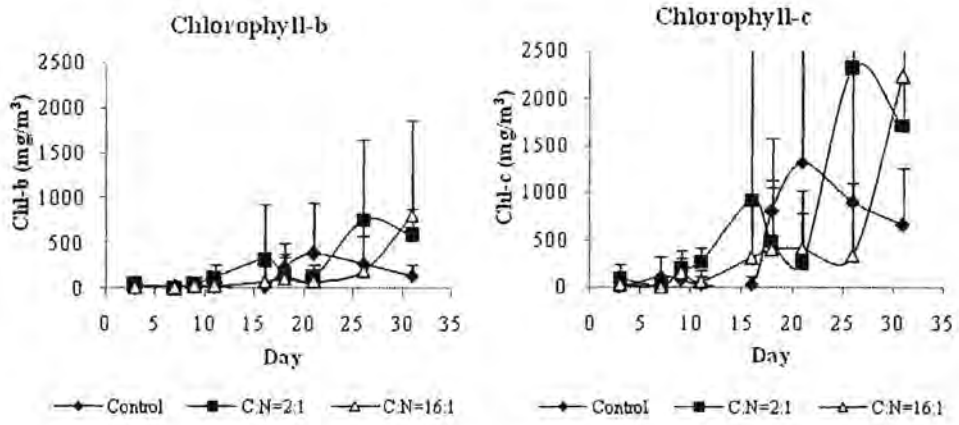


Fig. 4.7 Suspended solids and chlorophyll concentrations in water that was supplied with tapioca starch as a sole carbon sources and NH_4Cl/shrimp diets as combined nitrogen sources: (A) nitrogen sources were added to the control daily, (B) C:N ratio of 2:1 was added daily to treatment 1 and (C) C:N ratio of 16:1 was added daily to treatment 2.

Table 4.2 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. (°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 Zero-water exchanged tilapia cultivation in a biofloc system

4.2.1 Experiment 5: zero-water exchange tilapia cultivation in a biofloc system

Ranges of pH, temperature and alkalinity in control and treatment tanks were comparable and within acceptable range for practical aquaculture (Table 4.3). DO concentration was not a growth-limiting factor because it was maintained above 4.0 mg/L in all tanks by continuous aeration. Addition of alkalinity was necessary after day 45 in both control and treatment systems to prevent the pH from decreasing below 7.0. Table 4.4 indicates the growth performance of tilapia in control and treatment tanks. High survival rate (96%) was observed in treatment tanks. For control tanks, tilapia death occurred gradually until all fish in two out of three replicate tanks were dead after 5 weeks. Survival rate was only 22% for the remaining control tank. Tilapia in both control and treatment tanks exhibited the average daily growth rate at 1.0 and 1.4 g/day, respectively

Table 4.3 Physical parameters including temperature, alkalinity, and pH for the control and treatment tanks, values in parenthesis indicate range

Parameter	Controls	Treatments
Temperature (C)	29 ± 2	29 ± 2
	(26 – 33)	(26 – 33)
Alkalinity (mg/L $CaCO_3$)	120 ± 42	135 ± 26
	(75 – 185)	(100 – 175)
pH	7.7 ± 0.3	7.5 ± 0.2
	(7.4 – 7.8)	(7.1 – 8.1)

Table 4.4 Growth performance of fish in control and treatment tanks (average over each tank)

Parameter	Controls	Treatments
Individual initial weight (g)	32.2 ± 1.0	31.4 ± 0.8
Initial density (kg/m^3)	3.0	3.0
Individual final weight (g)	77.8 ± 3.1*	98.6 ± 5.8
Final density (kg/m^3)	1.56*	8.82 ± 0.52
Survival rate (%)	22*	96 ± 3
Average daily growth (g/day)	1.0*	1.4 ± 0.02

*Calculation using the data from control tank 3 only (fish in other control tanks had totally died after 5 weeks)

The result of water analysis indicated the sequential accumulations of TAN followed by nitrite and nitrate in both the control and treatment tanks (Fig. 4.8). In spite of the similarity in concentration profiles, TAN and nitrite accumulated at a slower rate in treatment tanks and had lower concentrations than those in control tanks. For control tanks, TAN reached the maximum concentration on day 14 (26 mg N/L) before declining and remaining under 0.5 mg N/L for the remainder of the experiment. The average TAN concentration in control tanks was 13 mg N/L during the period of TAN accumulation (i.e., day 1 to day 15). It required 24 days for TAN in treatment tanks to reach the maximum concentration (17 mg N/L) before declining and remaining under 0.5 mg N/L until the final day of the experiment. The average TAN concentration in treatment tanks was 12 mg N/L during the period of TAN accumulation (i.e., day 1 to day 29). An extended period of nitrite accumulation was noticeable after TAN concentration diminished in both systems. Nitrite was detectable in control tanks at a high level for 30 days (i.e., day 13 to day 42), averaging at 29 ± 12 mg N/L during that period. A shorter period of high nitrite concentration was observed in treatment tanks, extending for 24 days (i.e., day 24 to day 47) with the mean nitrite concentration at 26 mg N/L during that period. Effective TAN and nitrite control occurred after day 42 in control tanks when nitrate concentration increased along with negligible TAN and nitrite concentrations (i.e., under 0.5 mg N/L) for the remainder of the experiment. A similar result was observed in treatment tanks after day 47.

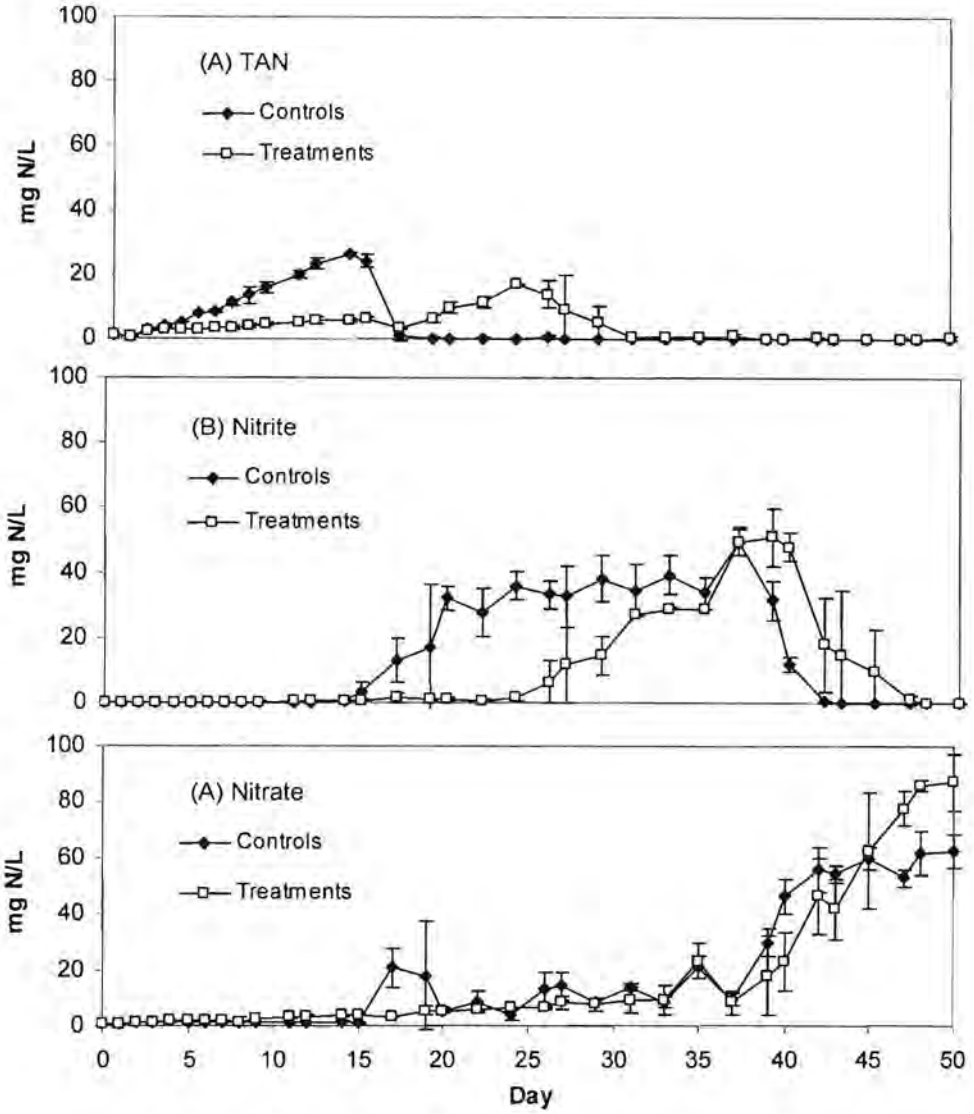


Fig. 4.8 Inorganic nitrogen profiles in the control and treatment tanks during the zero-water exchanged tilapia cultivation

Daily starch supplementation influenced the extent of biofloc formation (Fig. 4.9). Biofloc concentration, measured as total suspended solids, increased from 52 to 1,180 mg/L in treatment tanks receiving feed and tapioca starch while it remained relatively constant after day 22 at 99 ± 17 mg/L in control tanks receiving feed only. Biofloc volume was 16 and 86 mL/L on day 50 for control and treatment tanks, respectively. Biofloc CHN composition did not vary significantly between four sampling dates for each system (Fig. 4.10). Average hydrogen content was about the

same in both treatments. Statistical analysis indicated that the carbon and nitrogen contents of bioflocs differed significantly ($P < 0.05$) between control and treatment tanks (i.e., ≈ 4.5% dried weight). Average carbon content of bioflocs in control tanks (21.7 ± 3.1% dried weight) was approximately 60% less than that in treatment tanks (34.5 ± 0.9% dried weight) while average nitrogen content of bioflocs in treatment tanks (4.2 ± 0.4% dried weight) was nearly twice that in control tanks (2.2 ± 0.3% dried weight).

A nitrogen mass balance was performed by estimating the nitrogen input from feed and the nitrogen fate in the forms of dissolved inorganic nitrogen in water (i.e., TAN, NO_2-N, and NO_3-N), bioflocs, and tilapia (Table 4.5). Nitrogen in bioflocs was based on the results of the CHN analysis. Nitrogen in tilapia was assumed at 2.3% of the fish dried weight (Siddiqui and Al-Harbi 1999). Feed accounted for essentially all the nitrogen input in both control and treatment tanks. Nitrogen input to control tanks was less than that to treatment tanks because of high fish mortality in control tanks. Dissolved inorganic nitrogen in both treatments was present mainly as nitrate on day 50 while the amount of TAN and nitrite were insignificant. Recovery of nitrogen by tilapia in treatment tanks was 33%. A large proportion of nitrogen in both treatments could not be accounted for. In addition, 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.

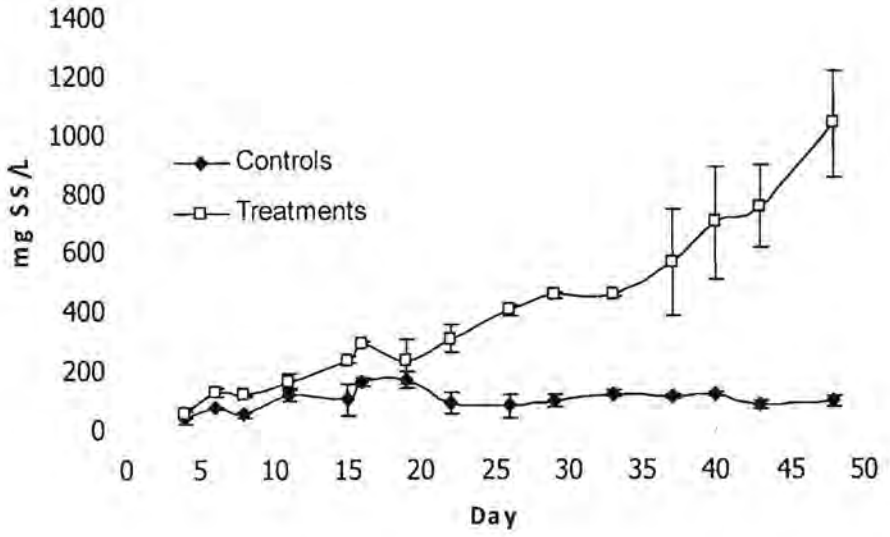


Fig. 4.9 Suspended solids concentrations in the control and treatment tanks during the zero-water exchanged tilapia cultivation

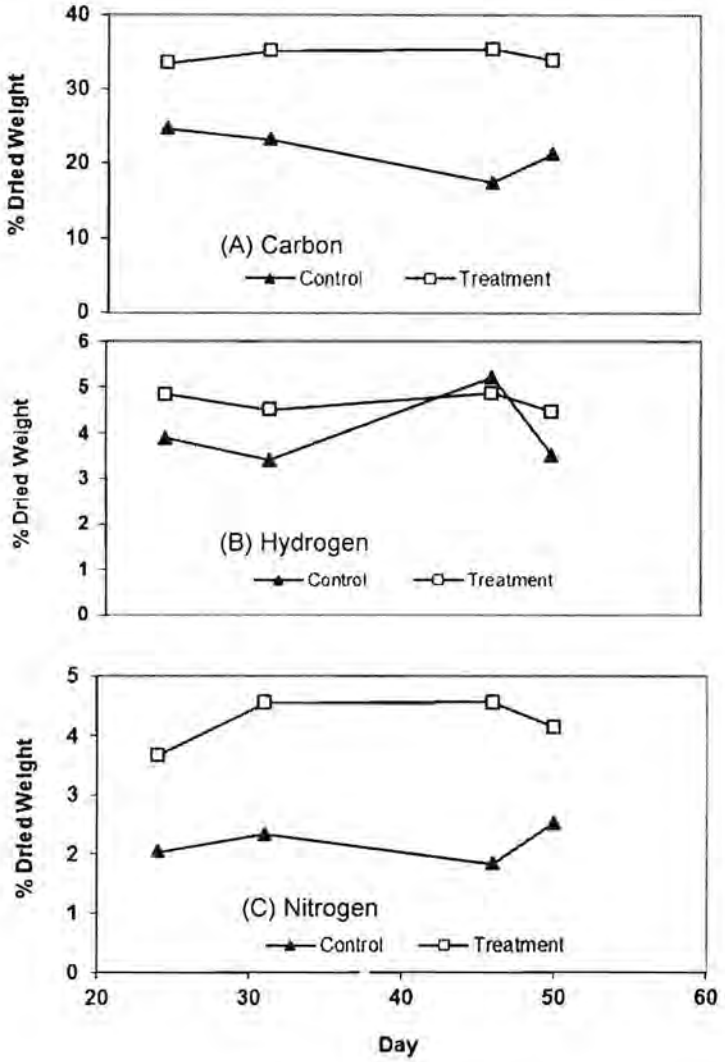


Fig. 4.10 CHN analysis of bioflocs in the control and treatment tanks during the zero-water exchanged tilapia cultivation (values are means of three replications)

Table 4.5 Nitrogen balance calculation displaying nitrogen distribution in the control and treatment tanks

Parameter (per tank)	**Control Tanks**	**Treatment Tanks**
Total input (g-N)	53.8 (100%)	199.8 (100%)
Feed (g-N)	53 (99%)	199 (> 99%)
Initial water (g-N)	0.8 (1%)	0.8 (< 1%)
Final Day (Day 50)		
TAN (g-N)	0.05 (< 1%)	0.23 (< 1%)
NO_2-N (g-N)	0.01 (< 1%)	0.12 (< 1%)
NO_3-N (g-N)	31.1 (58%)	43.7 (22%)
Biofloc (g-N)	2.2 (4%)	25 (13%)
Nitrogen gained in fish (g-N)	6.5 (12%)	66.6 (33%)
Unaccounted nitrogen (g-N)	14 (26%)	64.1 (32%)

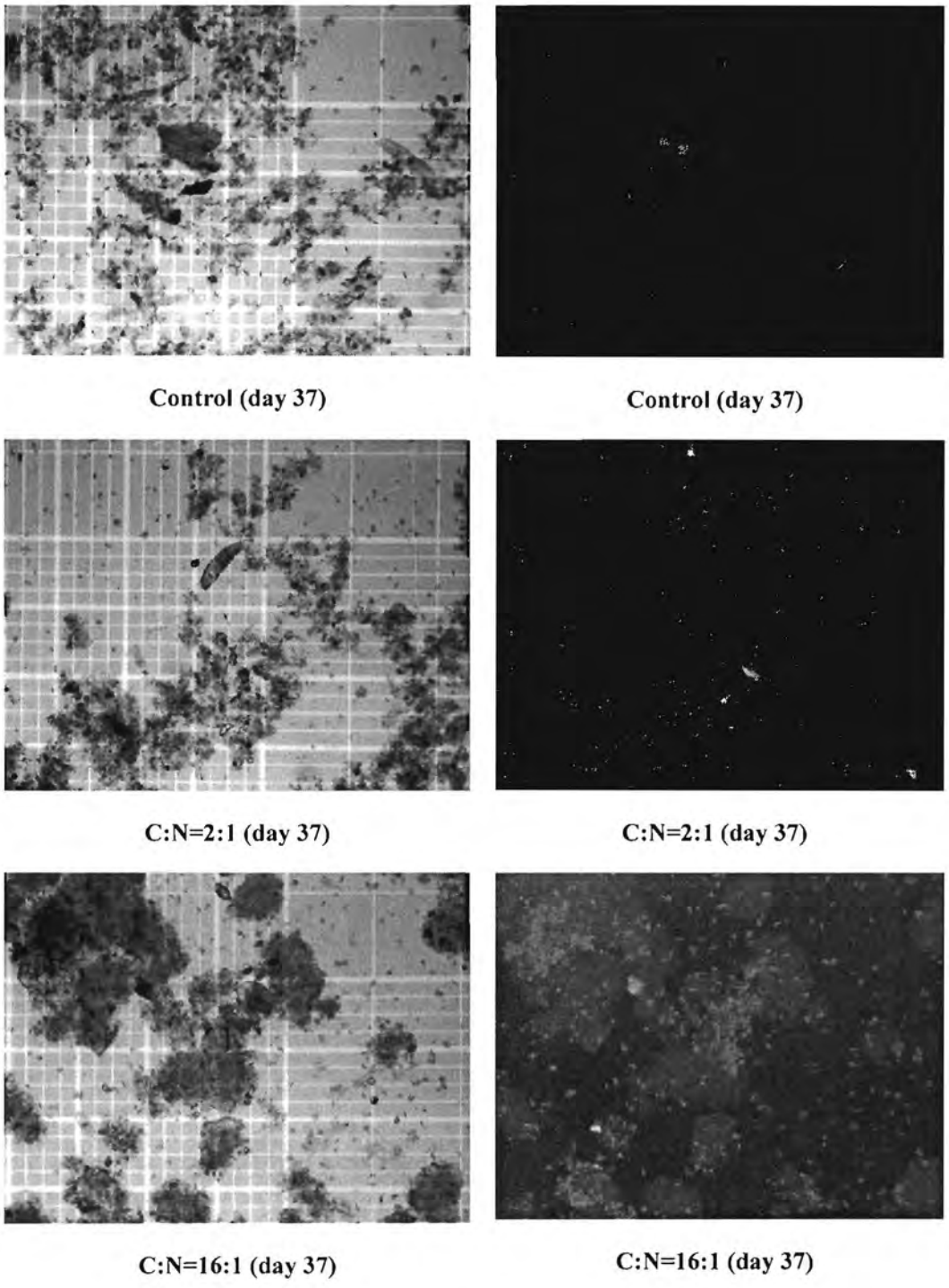


Fig. 4.11 Fluorescent microcopy of biofloc samples taken on day 37 from the controls (nitrogen only), treatment 1 (C:N = 2:1) and treatment 2 (C:N = 16:1

CHAPTER V
DISCUSSION

5.1 Optimal condition for biofloc formation

The impact of providing an organic carbon source to establish biofloc-mediated assimilation was examined in this study for its effectiveness in controlling inorganic nitrogen concentrations in a zero-water exchange aquaculture system. Biofloc technology is based on inorganic nitrogen assimilation into heterotrophic bacterial biomass. Inorganic nitrogen controls in biofloc systems can be accomplished by reducing feed protein contents to increase organic carbon availability in water (Hargreaves 2006; Azim et al., 2008) or adding inputs with the appropriate C:N ratio to stimulate heterotrophic bacterial growth (Avnimelech, 1999). Providing the organic carbon and nitrogen sources at the C:N ratio of 16:1 was the most effective in controlling inorganic nitrogen concentrations (Fig. 4.1 and 4.6). It is expected that using higher C:N ratios would result in greater amount of bioflocs and better inorganic nitrogen control, but the method may not be practical due to the need to provide good mixing and intensive aeration, which led to higher operational expense. The use of this particular C:N ratio (i.e., C:N = 16:1) resulted in a rapid increase of bioflocs relative to other treatment systems (Fig. 4.4 and 4.7) The chosen C:N ratio was also near the optimal range (i.e., C:N = 10:1 to 15:1) for maximum heterotrophic bacterial growth (Goldman et al., 1987; Lechevallier et al., 1991; Avnimelech, 1999; Hargreaves, 2006; Schneider et al., 2007; De Schryver and Verstraete, 2008). In this experiment, tapioca starch was employed to substitute glucose as the organic carbon source. Many organic compounds were possible in biofloc systems including starch, glucose, glycerol, acetate and molasses (Avnimelech, 1999; Avnimelech, 2006; Schneider et al., 2006; De Schryver and Verstraete, 2008). Literature reviews did not specify the characteristics of organic carbon that should be added to biofloc pond but seemed to emphasize on the cost and availability of organic carbon compounds on site.

Phytoplankton proliferation occurred in all treatments receiving organic carbon addition with the treatment subjected to C:N ratio of 16:1 showing the most rapid phytoplankton growth (Fig. 4.3). This observation was unexpected because literatures indicated the gradual population shift towards heterotrophic microorganisms as addition of organic carbon sources continued (Ebeling and Timmons, 2007). The reason for this observation (i.e., domination of autotrophic microorganisms) was likely linked to the duration of the experiment, which lasted for approximately 3 to 4 weeks. Tacon et al. (2002) observed the population shifted from autotrophic green microalgae towards heterotrophic microorganisms in intensive

shrimp cultivating ponds receiving low protein feed contents after 6 to 8 weeks. Similar result was reported for semi-intensive tilapia cultivating tank (NIA Report, 2008).

5.2 Zero-water exchanged tilapia cultivation in a biofloc system

Important conclusion can be drawn from the zero-water exchanged tilapia cultivation in a biofloc system. Tanks used in the experiment were covered with plastic lids to eliminate the effect of phytoplankton growth. This situation can be easily duplicated in intensive indoor aquacultures, which are becoming more popular due to better disease control, higher production yield, and environmental protection (Avnimelech, 2006, Sesuk et al., 2009). The addition of tapioca starch to treatment tanks did not significantly change the general trend of inorganic nitrogen concentrations from that in control tanks (Fig. 4.8). Theoretically, TAN, nitrite, and nitrate concentrations in biofloc systems should be stable and low as a result of inorganic nitrogen conversion into bacterial biomass (Ebeling and Timmons, 2007; Schneider et al., 2007). However, inorganic nitrogen profiles from both systems exhibited the sequential accumulation of TAN followed by nitrite and nitrate. Such profiles are common during the start-up of biofilters in recirculating aquaculture systems (Timmons et al., 2002; Hari et al., 2006; Kutako et al., 2009) and are characteristics of nitrification (Hargreaves, 1998; Sesuk et al., 2009; Xue et al., 2009). In this experiment, control and treatment tanks required approximately 6 to 7 weeks to establish the complete nitrification (Fig. 4.8). The lag in development of nitrification in treatment tanks (i.e., using time of peak ammonia as an indicator) can be explained by competition for ammonia substrate between heterotrophs and nitrifiers, especially at substrate-limiting concentrations (Malone et al., 2006). Organic carbon added to treatment tanks stimulates the growth of heterotrophic bacteria, which have a growth rate that is about ten times greater than nitrifiers (Tchobanoglous et al., 2003; Hargreaves, 2006; Schneider et al., 2006). Providing organic carbon (i.e., starch) favors heterotrophs and maintains lower TAN concentration, thereby extending the acclimatization period required for nitrifying bacteria activation, even under appropriate conditions for nitrification. Addition of tapioca starch to treatment tanks also resulted in higher carbon and nitrogen contents of bioflocs compared to those in control tanks (Fig. 4.10). Carbon and nitrogen are basic building blocks for cell synthesis. Providing organic carbon (i.e., starch) to treatment tanks accelerates heterotrophic bacteria growth and stimulates a more rapid nitrogen uptake compared to bioflocs in control tanks that received only feed addition.

Inorganic nitrogen profiles (Fig. 4.8) and nitrogen balance calculations (Table 4.5) suggest that assimilation and nitrification were important for inorganic nitrogen treatment in biofloc technology systems at different stages of the experiment. Prior to the establishment of complete nitrification in week 6 and 7, assimilation was the major pathway for inorganic nitrogen controls in treatment tanks. Though TAN and nitrite concentrations were undesirable (i.e., greater than 1.0 mg N/L) in both treatments, the addition of tapioca starch in treatment tanks resulted in a slower rate of

TAN and nitrite accumulations and lower TAN and nitrite concentrations relative to control tanks (Fig. 4.8). This observation and a significant increase of biofloc concentration in treatment tanks during the same period (Fig. 4.9) confirmed the importance of assimilation in the partial control of inorganic nitrogen toxicity during biofloc system start-up. A similar result was reported by Hari et al. (2006), where the addition of low protein feed and tapioca starch (i.e., C:N = 20:1) to extensive shrimp ponds resulted in significantly lower TAN and nitrite concentrations compared to adding only high protein feed during the 5-week period prior to nitrate accumulation. Supplying organic carbon to mediate nitrogen assimilating process was beneficial because it reduced TAN and nitrite concentrations in water, and this strategy might be employed as alternative measure to lower the unexpected increase of TAN and nitrite concentrations during system start-up. Effective controls of TAN and nitrite concentrations proceeded after complete nitrification was established regardless of organic carbon supplementation. The role of nitrification in maintaining inorganic nitrogen concentrations in control tanks was apparent as can be seen by the complete oxidation of TAN and nitrite to nitrate for the remaining of the experiment (Fig. 4.8). The complete oxidation of TAN and nitrite by nitrifiers in treatment tanks receiving organic carbon addition was favored by high oxygen concentration, suitable alkalinity, and bioflocs and tank walls likely served as attachment sites for slow-growing nitrifying bacteria.

The nitrogen mass balance calculation (Table 4.5), showing significant amount of nitrogen accumulation in the form of nitrate (58%), confirmed the important role of nitrification in control tanks. For treatment tanks, the majority of nitrogen conversion was distributed as nitrate and bioflocs (Table 4.5). Higher amount of nitrogen in nitrate (22%) compared to bioflocs (13%) indicated that nitrification was more important than assimilation over the long term. Therefore, nitrification, and to lesser extent assimilation, were both responsible for effective controls of inorganic nitrogen concentrations. Significant nitrifying activity in biofloc systems was also described by Azim and Little (2008). In that work, nitrate concentration in tilapia tanks reached 250 mg N/L after 11 weeks despite the tanks being seeded with 350 mg/L of bioflocs and fed daily with low protein (24%) diets. Simultaneous occurrence of assimilation and nitrification occurs in many activated sludge process units that treat domestic and industrial wastewater (Charley et al., 1980; Tchobanoglous et al., 2003).

The proportion of nitrogen that could not be accounted for was significant in both systems (Table 4.5). The magnitude of nitrogen loss in this experiment was similar to Thakur and Lin (2003), who reported nitrogen loss as high as 36% during zero-discharged shrimp cultivation in concrete tanks. Nitrogen loss as high as 55% was reported in brackish ponds with very limited water discharge (Daniels and Boyd, 1989). Denitrification and ammonia volatilization were assumed to be the pathways for nitrogen loss (Thakur and Lin, 2003; Hari et al., 2006). Ammonia volatilization was not expected to be significant because TAN was less than 1.0 mg N/L and the pH was between 7 and 8 so that the major fraction of TAN was in the soluble ionized form (i.e., NH_4^+-N). Nitrogen loss via denitrification was more likely in this

experiment due to the high level of nitrate in water, the availability of dissolved organic carbon, and the presence of anaerobic pockets at the inner region of biofloc or caused by biofloc sedimentation on tank bottoms.

Slow fish growth could be linked to chronic toxicity of high TAN and nitrite concentrations and high suspended solids concentration (Hargreaves and Kucuk, 2001; Timmons et al., 2002). Generally TAN and nitrite concentrations less than 1.0 mg N/L are recommended for long term exposure (Timmons et al., 2002). Severe growth inhibition and increased mortality of tilapia were observed when the suspended solids concentration exceeds 850 mg/L (Little et al., 2008). Thus, maintaining optimal suspended solids concentration may be the critical aspect in managing biofloc technology systems, with the recommended maximum suspended solids concentration at 500 mg/L (Avnimelech, 2006; Azim and Little, 2008; Little et al., 2008). Solids removal was not conducted in this study, resulting in excessive suspended solids concentration (i.e., > 500 mg/L) that may have reduced visibility and consequently the ability of tilapia to find feed (Azim and Little, 2008).

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

1. Organic carbon addition at high C:N ratios was more effective in maintaining TAN and nitrite concentrations in comparison to lower C:N ratios. The daily addition of carbon and nitrogen at the C:N ratio of 16:1 resulted in the most effective inorganic nitrogen control and highest biofloc formation. Direct assimilation of inorganic nitrogen by phytoplankton and heterotrophic bacteria was likely the main mechanisms that were responsible for inorganic nitrogen control and biofloc formation. The occurrence of nitrification also exerted the positive impact on water quality as it converted TAN and nitrite to nitrate. 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. The result of the zero-water exchanged tilapia cultivation demonstrates the role of nitrogen assimilation and nitrification in the control of inorganic nitrogen levels in a biofloc aquaculture system. The result indicated that assimilation and nitrification were important for inorganic nitrogen control at different stages of the experiment. In the first stage, prior to the occurrence of complete nitrification, addition of organic carbon promoted nitrogen assimilation into microbial flocs. In the following stage, nitrification and to a lesser extent assimilation were both responsible for effective inorganic nitrogen control. Based on the results, further study is necessary to identify ecological relationships between nitrifying and heterotrophic bacteria in biofloc systems. The addition of organic carbon may be conducted until the establishment of complete nitrification or used as a strategy to quickly reduce TAN and nitrite concentrations. Biofloc management strategy, specifically the development of an effective biofloc separator, is necessary to improve the efficiency and sustainability of biofloc aquaculture systems.

REFERENCES

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.

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.

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.

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.

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.

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.

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. Bioresource Technology 98, 1700-1703.

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

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

Hargreaves, J. A., 2006. Photosynthesis suspended-growth systems in aquaculture. Aquaculture engineering 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. Aquaculture 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.

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.

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.

NIA Research Report (2008), Development of biofloc and nitrification system for tilapia cultivation, National Innovation Agency, Thailand.

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

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

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

Strickland, J.D.H. and Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis. (2nd 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.

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.

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.

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

APPENDIX - OUTPUT

International publication

Nootong, K., Pavasant, P., and Powtongsook, S., 2011. Effect of organic carbon addition in controlling inorganic nitrogen concentrations in a biofloc system. Journal of the World Aquaculture Society. (Under Production – Expected online June 2011)

International Conference

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

National publication

Vanitchanai, W., Powtongsook, S., and Nootong, K., 2008. Effect of organic carbon addition in controlling inorganic nitrogen toxicity for the closed-water aquaculture application. *In Proceeding 34th Congress on Science and Technology of Thailand*, Queen Sirikit National Convention Center, Bangkok, Thailand October 31 – November 2008.

Vanitchanai, W., Powtongsook, S., and Nootong, K., 2009. Effect of organic carbon addition on inorganic nitrogen control and biofloc characteristics during the closed-water tilapia growout in suspension system. *In Proceeding 35th Congress on Science and Technology of Thailand*, The Tide Resort, Chonburi, Thailand, October 15 – 17, 2009.

Master Thesis

Effect of organic carbon addition on microbial floc formation and water quality in closed aquaculture system, Master Thesis in Chemical Engineering (2009), Chulalongkorn University (ระดับ ดีมาก)