Chapter III

Materials and Methods

This research is structured into two main sections. The first section describes the possible strategies for nitrifying biofilter acclimation and physical factors affecting nitrification startup. The second section evaluates the performance of the proposed aquaculture system integrated with acclimated nitrifying biofilters in controlling inorganic nitrogen toxicities.

3.1 Strategies for Nitrifying Biofilter Acclimation

3.1.1 Parameters Affecting Nitrification

1. Types of ammonium sources. Ammonium from shrimp diets, NH_4Cl, and tilapia excretion were used to compare their effects on nitrifying rates and required duration to establish the complete nitrification.
2. Ammonium concentrations. Different ammonium concentrations at 2 and 10 mg N L^{-1} from shrimp diets and NH_4Cl were used to compare their effects on nitrifying rates and required duration to establish the complete nitrification.
3. Types of biofilters. Two types of commercially available biofilters, Biocord™ and BCN-009, were used to compare their effects on nitrifying rates and required duration to establish the complete nitrification.

The experiment in section 3.1.1 was conducted in 10 sets of plastic containers (volume 120 L) fill with tap water, which was continuously aerated for about a week to remove Chlorine residues. Aeration for biofilters was provided by using two diffusive stone aerators to keep DO concentration greater than 4 mg L^{-1}. The pH and alkalinity were maintained in the range from 7 – 8 and from 100 – 150 mg L^{-1} $CaCO_3$, respectively. Deviations of these parameters from specified ranges were adjusted by adding $CaCO_3$. Water samples from each experimental tank were collected daily to

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analyse for NH_4^+-N, NO_2^--N, and NO_3^--N concentrations accoding to the Standard Methods (1998).

3.1.2 Biofilter Acclimation for Zero-Water Exchanged Tilapia Cultivation

This experiment was performed in parallel to section 3.1.1. The main purpose of this subsection was to prepare nitrifying biofilters for future use during an evaluation of the proposed aquaculture systems. Commercial fibrous Biocord™ biofilters (polypropylene; specific surface area: 2.8 m^2 m^{-1} or 82.35 m^2 kg-biofilter^{-1}) were cut into 30 pieces (60 cm each), and fixed with weighling stones to ensure the total submergence under water in a 1,000 L plastic acclimating tank. Approximately 25.0 g of 37 ± 2% protein shrimp diets were grounded and added into an acclimating tank filled with 800 L water to provide the initial dose of ammonium concentration at 1.85 mg N L^{-1}. About 2.0 g of sediments from a Pacific white shrimp cultivating tank in the same laboratory were also added into acclimating to supply mixed nitrifying seeds. A black plastic cover was placed over the top of the acclimating tank to prevent rainwater and sunlight from promoting growth of phytoplankton. Acclimation of Biocord™ biofilters was carried out in acclimating tank without water exchange for 78 days. Water samples, taken at least four times a week from acclimating tank, were analyzed for NH_4^+-N, NO_2^--N, and NO_3^--N concentrations accoding to the Standard Methods (1998). Identical amounts of shrimp diets (25 g) were replenished in the acclimating tank once an ammonium concentration in water was undetectable. In order to examine the ability of acclimated biofilters to sustain nitrification at higher ammonium loadings, the shrimp diets were replaced by 9.17 and 13.76 g of an analytical graded NH_4Cl on day 64th and 71st, respectively. In this experiment, a completely mixed hydraulic regime in acclimating tank was maintained by a constant aeration to provide DO > 4.0 mg L^{-1}. Alkalinity and pH were controlled at between 100 and 150 mg L^{-1} $CaCO_3$ and from 7 – 8.2 by adding $NaHCO_3$. SEM examination was also carried out to confirm microbial attachment on the surface of Biocord™ biofilters.

3.1.3 Determination of Nitrification Rate

Small pieces (10 – 15 cm) of acclimated Biocord™ from section 3.1.1 and 3.1.2 as well as a portion of BCN-009 (150 pieces) were taken to perform batch

experiments to determine nitrification rates. Batch experiments were performed at the initial ammonium concentrations of 2, 4, 8 and 12 mg N L^{-1}. For each initial ammonium concentration tested, batch experiments were carried out in two replicates in 6 L plastic bottles equipped with a stone aerator to provide thoroughly mixed conditions and DO > 4 mg L^{-1}. Alkalinity and pH were maintained at between 100 and 150 mg L^{-1} $CaCO_3$ and from 7 – 8.2, respectively. Approximately 9 mL of water from 6 L plastic bottles were collected at predetermined intervals and later analyzed for NH_4^+-N, NO_2^--N, and NO_3^--N concentrations accoding to the Standard Methods (1998).

3.2 Performance Evaluation of the Proposed Aquaculture Systems

3.2.1 Preparation of Cultivating System

Figure 3-1 illustrates the sketch of the proposed aquaculture systems. A circular plastic tank (500 L) was employed to accommodate the acclimated biofilters described in section 3.1.2. Acclimated biofilters were completely submerged under the water surface within a hollow cylindrical plastic net (inner diameter = 30 cm, outer diameter = 30.6 cm and height = 90 cm), which was entirely wrapped in thin plastic sheet except for the top and bottom ends. Acclimated biofilters located inside the net were connected to a metal frame lying on tank floor to ensure that acclimated biofilters were able to align vertically. Within this net, the acclimated biofilters were free from fish interferences and were fully oxygenated by diffusive stone aerator to provide upflow water movement by means of airlift actions. Water circulation between inside and outside the nets was made possible by making a small opening (width = 1 cm and length = 8 cm) as water outlet on thin plastic sheel about 0.5 – 1.0 cm above water surface. Aeration of biofilters also served to maintain aerobic conditions for the aquatic stocks. Additional aeration outside biofilter net could be installed to ensure good animal welfare. Clearly, the proposed aquaculture ystem was different from the conventional designs, which normally located the treatment unit (i.e., biofilters) outside production ponds. In this study, acclimated biofilters were installed in the same tank as aquacultures so that the rearing of aquactic stocks, water treatment and separation of suspended solids were able to be performed simultaneously.

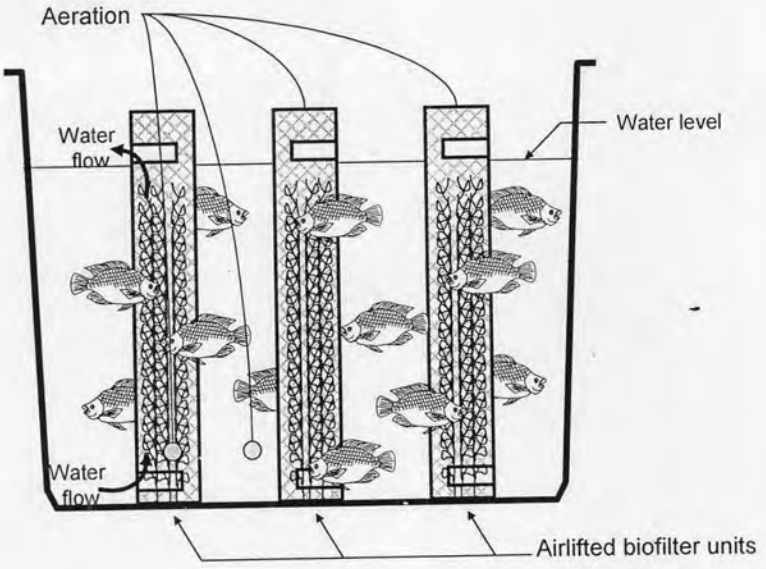

Figure 3-1 Sketch of the proposed aquaculture system (Not to scale)

3.2.2 Experimental Animals

Tilapia (*Oreochromis niloticus*) was used during the zero-water exchanged cultivation experiment. Male tilapia weighed from 30 – 100 g fish^{-1} was kept in preparation tank (volume 1,000 L) for about 1 – 2 weeks for observation. Healthy tilapia from preparation tank was selected and then stocked in the proposed aquaculture systems. Protein content in tilapia feed was fixed at 30%. Feeding was performed twice a day in the morning (7:00 am) and in the evening (5:30 pm) at 3% of total fish weight throughout the experiment.

3.2.3 Evaluation of the Proposed Aquaculture Systems at Different Initial Tilapia Stocking Density

3.2.3.1 Initial Stocking Density at 0.7 kg m^{-3}

The zero-water exchanged tilapia cultivation at initial stocking density of 0.7 kg m^{-3} was carried out in the proposed aquaculture systems similar to Figure 3-1. In this case, contruction of the proposed aquaculture systems followed the description detailed in section 3.2.1 except that only one cylindrical plastic net was installed in each tank. Acclimated Biocord^{TM} biofilters were from section 3.1.2. The total length of acclimated Biocord^{TM} biofilters installed in each tank was approximately 10 m.

Tilapia with average initial weight of 116 ± 3.96 g were stocked in four replicated sets of cultivating tanks to produce an average initial biomass density for each tank at 772 ± 26.41 g m^{-3}. Tilapia growth data was determined by measuring weights and lengths of tilapia every 3 weeks. Tank 1 and 2 (T1 and T2) were two replicated sets of the proposed aquaculture systems, which integrated acclimated Biocord™ biofilters described in section 3.1.2. Tank 3 (T3) featured no Biocord™ biofilters. Tank 4 (T4), arranged with non-acclimated (new) Biocord™ biofilters, was constructed following the scheme of the proposed aquaculture system. Dissolved oxygen concentration was kept at least 4.0 mg L^{-1} by installing 4 diffusive stone aerators (2 aerators in the plastic net; 2 aerators outside the plastic net). Hydraulic regime was completely mixed while alkalinity and pH were maintained at the optimal range for nitrification (i.e., pH = 7 – 8; alkalinity = 100 – 150 mg L^{-1} $CaCO_3$). All cultivating tanks were located outdoor under heavy shade. Analysis for NH_4^+-N, NO_2^--N, NO_3^--N, and suspended solid concentrations were constantly monitored according to the Standard Methods (1998).

3.2.3.2 Initial Stocking Density at 3.0 kg m^{-3}

Based on the result from section 3.2.3.1 (i.e., initial stocking density at 0.7 kg m^{-3}), it was necessary to further assess the proposed aquaculture systems at higher stocking density. In this section, the zero-water exchanged tilapia cultivation at the initial stocking density of 3.0 kg m^{-3} was carried out. The proposed aquaculture systems used circular plastic tanks (450 L working volume) to accommodate acclimated Biocord™ biofilters and aquacultures. The design of the proposed aquaculture systems was identical to Figure 3-1. Acclimated Biocord™ biofilters (length 7.2 m) were completely submerged under the water surface within a hollow cylindrical plastic net (inner diameter = 30 cm; outer diameter = 30.6 cm; height = 90 cm), which was entirely wrapped by a thin plastic sheet except for the top and bottom. Total of three plastic nets were used in each proposed system to give the total biofilter length of 21.6 m (i.e., ≈ 60.5 m^2). In this section, tilapia with an average initial weight of 56 ± 6.5 g was stocked in 450 L plastic tanks and grew without any water exchange for 99 days. T1 and T2 were replicated experimental systems that were fabricated following the design shown in Figure 3-1. Initial tilapia stocking density for these tanks was at 3.4 kg m^{-3}. T3, initially stocked with 1.7 kg m^{-3} of tilapia, was another experimental system built to resemble Figure 3-1. Finally, T4 was a regular fish tank

that featured no Biocord™ biofilters and initially stocked with tilapia at 3.2 kg m^{-3}. The experiment was located outdoor under heavy shade. Weight and length of tilapia were measured about every 3 weeks. Analysis for NH_4^+-N, NO_2^--N, NO_3^--N, and suspended solid concentrations in water columns were constantly monitored according to the Standard Methods (1998). Hydraulic regime was completely mixed. Other operating conditions were maintained as followed; DO > 4 mg L^{-1}, pH = 7 – 8, and alkalinity = 100 – 150 mg $CaCO_3$ L^{-1}.

3.2.3.3 Initial Stocking Density at 5.0 kg m^{-3}

Additional experiment was carried out at the initial tilapia stocking density of 5.0 kg m^{-3}. In this case, the effects of biofilter cleaning and outdoor cultivation were assessed. The description of the proposed aquaculture system was identical to section 3.2.3.2. Tilapia with an average initial weight of 56 ± 6.5 g was stocked in 450 L plastic tanks and grew without any water exchange for 99 days. Tank 1 (L1), located outdoor and subjected to biofilter cleaning every 2 weeks, was fabricated following the design of the proposed aquaculture system. Tank 2 (L2) was identical to L1 except no biofilter cleaning was taken place. Tank 3 (D1) was identical to L1 except the system was located in the dark. Tank 4 (D2) was identical to D1 except no biofilter cleaning was done. It should note that L2 and L1 were subjected to full sunlight only in the morning between 8:00 – 10:00 am. Weight and length of tilapia were measured about every 3 weeks. Analysis for NH_4^+-N, NO_2^--N, NO_3^--N, and suspended solid concentrations in water columns were constantly monitored according to the Standard Methods (1998). Hydraulic regime was completely mixed. Other operating conditions were maintained as followed; DO > 4 mg L^{-1}, pH = 7 – 8, and alkalinity = 100 – 150 mg $CaCO_3$ L^{-1}.

3.2.3.4 Biofilter Solid Retention

During the experiment in section 3.2.3.1 (i.e., 0.7 kg m^{-3} initial stocking density), a significant amount of suspended solids was observed to be retained on the surface of Biocord™ biofilters. Therefore, an independent experiment was set up to demonstrate biofilter ability in retaining solids. Two replicated sets (10 – 15 cm) of unused and acclimated Biocord™ biofilters were subjected to 4.5 L of turbid water containing approximately 800 mg SS L^{-1}. Aeration by a single diffusive stone aerator was provided normally for both unused and acclimated Biocord™ biofilters. Water

turbidity was regularly monitored at predetermined intervals by measuring optical density (OD) at 660 nm.

3.3 Water analysis

3.3.1 Ammonium (NH_4^+-N)

Ammonium concentration was analyzed by phenol-hypochloride reaction according to the Standard Methods (1998). Five milliliters of filtered water sample diluted with deionized water was mixed with 0.2 mL of phenol solution (dissolve 20 g of crystalline analytical grade phenol in 200 mL of 95% (v/v) ethyl alcohol), 0.2 mL of sodium nitroprusside solution (1 g of $Na_2Fe(CN)5NO.2H_2O$ in 200 mL of deionized water), and 0.5 mL of freshly prepared oxidizing reagent (100 g of sodium citrate and 5 g of sodium hydroxide in 500 mL of deionized water mixed with sodium hypochloride 4:1 (v/v)) respectively. After mixing and stand for 1 hour, absorbance of the solution was measured at 640 nm using spectrophotometer (GENESIS®; model 10 UV scanning) against the blank (de-ionized water). Ammonium concentration (mg N L^{-1}) was calculated using the standard curve of ammonium solution (0.01 – 1.00 mg N L^{-1}).

3.3.2 Nitrite (NO_2^--N)

Nitrite was analyzed by sulfanilamide reaction according the Standard Methods (1998). Five milliliters of diluted water sample was mixed with 0.1 mL of sulfanilamide solution (dissolve 5 g of sulfanilamide in a mixture of 50 mL of concentrate hydrochloric acid and about 300 mL of distilled water) and 0.1 mL of (1-Naphthyl)-ethylenediamine dihydrochloride solution (0.5 g of N-(1-Naphthyl)-ethylenediamine dihydrochloride in 500 mL of distilled water). After mixing and stand for 1 hour, absorbance of the solution was measured at 543 nm using spectrophotometer (GENESIS®; model 10 UV scanning) against distilled water blank. Concentration of nitrite (mg N L^{-1}) was calculated using the standard curve of nitrite solution (0.007 – 0.224 mg N L^{-1}).

3.3.3 Nitrate (NO_3^--N)

Nitrate concentration was analyzed by UV screening method according to Standard Method (1998). Water sample was filtered through GF/C filter and measured directed by UV visible spectrophotometer at 220 and 275 nm. Calculation of nitrate concentration was as following:

$$\text{Nitrate (mg N L}^{-1}) = \frac{(Abs_{220nm} - Abs_{275nm}) \times A}{B}$$

where A = concentration of nitrate in standard curve (mg N L^{-1})

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

Standard nitrate solution was prepared using 1 – 10 mg N L^{-1} of sodium nitrate. It has to be noted that, this method must be strictly used with nitrate concentration between 1 – 10 mg-N L^{-1}. Water sample containing high nitrate concentration over 10 mg N L^{-1} must be diluted with de-ionized water prior to the analysis but water containing low nitrate concentration below 1 mg N L^{-1} was not applicable with this method. Moreover, high nitrite concentration than 10 mg N L^{-1} can interfere with nitrate measurement hence concentration of nitrate must be subtracted with nitrite concentration.

3.3.4 Alkalinity

Alkalinity of water (mg L^{-1} $CaCO_3$) was measured based on titration method by using a commercial test kit produced by the Faculty of Veterinary Science, Chulalongkorn University.

3.3.5 Dissolved Oxygen

Dissolved oxygen (mg L^{-1}) in water column was monitored by using DO meter with integrated data logger (HI91410, Hanna, Portugal).

3.3.6 Temperature and pH

Monitoring of pH and temperature in water column was performed in *situ* by using pH meter (pHtestr30, Eutech Intrument, USA)

3.3.7 Total Suspended Solids

Total suspended solids were measured on basis of total solids dried at 103 – 105 °C according to Standard Method (1998). Dried Whatmann GF/C filter (47 mm) was placed in an oven overnight at 103 – 105 °C then stored in a desiccator to a constant weight (A). Pour a known volume of liquid sample (C) containing suspended solids through dried GF/C filter. The residue retained on the filter paper was dried in the temperature controlled oven overnight at 103 – 105 °C then stored in a discicator to a constant weight (B). The concentration of total suspended solids can be calculated as:

$$\text{Total suspended solids (mg SS L}^{-1}) = \left(\frac{(A-B)\times 1000}{C}\right)$$

where A is the weight of filter paper plus dried residue, mg.

B is the weight of filter paper, mg.

C is the volume of filtered liquid sample, mL

3.4 Tilapia Growth Data

Approximately 30% of total fish population was sampling once every 3 week. The following calculations were used to determine tilapia growth data during the zero-water exchanged cultivation.

$$\text{Average length (cm fish}^{-1}) = \frac{\text{Sum of total length}}{\text{Number of fish}}$$

$$\text{Average weight (g fish}^{-1}) = \frac{\text{Sum of total weight}}{\text{Number of fish}}$$

$$\text{Average daily growth (g day}^{-1}) = \frac{\text{Final weight per fish} - \text{Beginning weight per fish}}{\text{Cultivating period}}$$

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

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