Nitrogen Flow in a Recirculating Operation of *Litopenaeus vannamei* Maturation in Ecuador

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**Abstract**

The nitrogen flow together with dissolved nutrients and other parameters are described in a large shrimp maturation operation in Ecuador. In summary, only 8.9% of the nitrogen input ended in animal tissue. Nitrate-N and dissolved organic nitrogen (DON) accounted for more than 95% of the nitrogen pool. The nitrogen dynamics in the system is driven by a nitrification based trickling filter attached population and a free living heterotrophic carbon limited in the sump. The average nitrification rate of TAN (total ammonia nitrogen) averaged 44.81 gd⁻¹. The trickling filters worked as a nitrifier body and also reducing the organic load. There is also some evidence that the trickling filters might have also play some role in denitrification. Nitrate never reached high concentrations (<3.7 mg L⁻¹ nitrate-N), hence did not present a danger to the productivity of the system. In general either inorganic or organic phosphorous did not undergo any major change during the study. BOD values in the sump were low averaging 1.1 mg L⁻¹ more likely as a result of the carbon limitation of the system. A combination of low C:N ratio and high load of organic nitrogen in the trickling filter could be part of the key factors running this system. The semi closed recirculating system presented in this study has been used since 2004 providing a steady yield of nauplii without the seasonal effects typical of open system and without the accumulation of deleterious amount of waste nitrogen or the presence of pathogens. The present study demonstrates the feasibility of using a simple and inexpensive semi closed recirculating systems in a large commercial scale.

**Keywords**: *Litopenaeus vannamei*; Semi closed recirculating system; Maturation; Nitrification

**Abbreviations**: BOD: Biochemical Oxygen Demand; GSI: Gonadosomatic Index

**Introduction**

Penaeid culture practices have evolved from the simple systems described by Hudinaga [1] and Cook and Murphy [2] to high density floc and recirculating aquaculture systems (RAS) [3-7]. During the 70's, Mock and coworkers [8,9] were pioneers in describing and designing RAS for penaeid culture. At that time, this system may have been considered expensive and non-needed as there were few if any environmental regulations, major disease challenges or contamination issues which affect open systems. Since there, successful reproductive activity by *Litopenaeus vannamei* in completely closed systems have been reported [10,11]. These results demonstrated that closed systems can support reproductive performance at commercially acceptable levels.

Currently, RAS has become a priority, reasons for this; access to coastal areas; cost of land; runoff affecting water source, waste water regulations, and so on. In addition, because of their close nature of RAS, the risk of disease is also reduced. RAS typically include fish, shrimp or mollusk rearing tanks sustained by a water treatment process, which is focused on the detoxification of nitrogenous wastes, oxygenation, removal of suspended solids, and typically water exchange does not exceeds 10% [12,13].

Wastewater effluent from RAS comprises mainly feces, uneaten food and bacteria biomass, all of them rich in organic nutrients, minerals and vitamins. Heterotrophic bacteria are capable of taking up a variety of dissolved organic nitrogen (DON) compounds that may be sources of both nitrogen and carbon for biosynthesis or may also be used as sources of energy. Nutrients, released from the microbial activity include ammonia, nitrite and nitrate.

The accumulation of inorganic nitrogen and organic waste products in intensive aquaculture systems is one of the major limiting factors preventing further intensification. TAN (total ammonia nitrogen) and nitrite can be toxic to shrimp and accumulates not only through excretion of ammonia but also by the breakdown of organic solids. One important obstacle in RAS systems could be the accumulation of nitrate. High nitrate concentrations must be prevented as high concentration can affect shrimp. A detailed review of the nitrogen biogeochemistry and its process in aquaculture has been carried out by several researchers [14-20].

All the sea coastal countries around the world are aware of the need of increasing their knowledge of how the variations of oceanographic conditions influence the composition, distribution and abundance of the living resources of the sea [21,22]. These fluctuations have been documented in different topics. For instances, changes associated to the composition and abundance of the algae osmoprotectant dimethylsulfoniopropionate (DMSP), the primary productivity, penaeid shrimp larvae, groundfish species and the recruitment of fish stocks [23-27].

In this context, the Pacific Ocean off the coast of Ecuador is characterized by two differentiated seasonal periods; rainy summer (January to April) and a dry winter (July to October). In summer, a
southward coastal flow of warm low nutrient concentration and salinity water from the Panama Bight is dominant. Whereas, during the winter this mass of water is displaced north, in response to a strengthening of the trade winds and the richer, colder and higher salinity water mass of the Humboldt Current [21]. Additionally, the coast of Ecuador is also characterized by the periodical occurrence of “El Niño”. During this, anomalously high temperatures are observed in the upper layer of the ocean. An equally important event is that of “La Niña” which is characterized mainly by sea temperatures that are below the mean value and also has repercussions on the distribution of both coastal and oceanic resources [28].

Along the coast of Ecuador, regardless of the type of fluctuation, oceanographic changes impact both nauplii and larvae production. For example, egg and nauplii production per female ranged from 200,000 and 110,000 respectively per female in the steady months, in contrast, these values sharply dropped (up to 40%) during seasonal changes. Consequently, there was a need to evaluate a semi closed recirculating system to stabilize production. The present study describes the design, operation and the nitrogen dynamics of a semi closed recirculating system initiated in 2004 and has since enabled the continuous production of higher rates and more stable production for almost 8 years, with an annual production averaging of 12 billion nauplii and 1.2 billion post larvae.

Materials and Methods

The maturation unit

The maturation unit studied consist of 25 circular and 6 rectangular tanks (5.78 and 18.93 m³ of volume respectively, 258 m³ of water) holding a total of 3,190 broodstock of *L. vannamei*, 1,487 females (average weight 40.8 g ± 4.02) and 1,703 males, (with an average weight of 36.56 g ± 2.0). Animals were fed six equal doses a day at 2 am, 9 am, 11 am, 1 pm, 6 pm and 11 pm with 30 Kg of fresh frozen food, which consist of 54.5% of a mix of squid, mussels and oysters, 22.7% of adult Artemia biomass, 13.6% of squid and 9.2% of bloodworms. Daily mortality was determined by picking out and registering dead animals once a day.

Growth rate and gonadosomatic index (GSI) was determined in marked animals. In order to avoid effects on mating, growth rate was determined in the non-matured population. GSI=[gonad weight×100]/Body Weight-1. The nitrogen retention was calculated using data from the non-matured population and including the GSI from the mating population, assuming that protein concentration of the ovaries was 18% [29]. Daily both eggs and nauplii were separately collected, rinsed using a 100 µm nylon mesh, and distributing them into 6×15 Liters buckets. Total count was determined by calculating the average from 10 samples counts per bucket using 1 mL pipette. The semi closed recirculating systems consist of 95 MT (metric ton) sump, whereas the maturation tanks were aerated by a 10 HP blower using air stones inside airlifts placed in each tank.

An average of 18.6% of the water from system (Figure 1A) was exchanged daily from 6:00 am to 3 pm with fresh sea water to offset evaporation, siphoning, movement of animals, tank harvest or detoxifying the system. Every day 2 Kg of sodium bicarbonate were dissolved into 20 Liters using water from the sump and added to the sump at dusk at a rate of 7 Liters per hour to compensate for any loss in alkalinity due to nitrification.

The trickling filter system consists of 8 circular glass towers, each tower with 4 levels of dishes (160 cm of diameter×10 cm of height). All dishes are flat bottom with hundreds of small holes so water cascade down as shower from dish to dish. First level from every tower had no stones substrate media, but is the level in which the water sprinkles homogeneously to the lower levels. In the three lower levels, 154.2 kg of stones per level are evenly distributed along the surface of the dishes (average height 4 cm). Animals were fed six equal doses a day at 2 am, 9 am, 11 am, 1 pm, 6 pm and 11 pm with 30 Kg of fresh frozen food, which consist of 54.5% of a mix of squid, mussels and oysters, 22.7% of adult Artemia biomass, 13.6% of squid and 9.2% of bloodworms. Daily mortality was determined by picking out and registering dead animals once a day.

Experiments A and B

**Nitrogen conversion analysis:** The rationale of this experiment was to evaluate the trickling filter as a nitrification and filtration treatment...
unit and to find out differences produced by the organic load in the sump found during the day. Water samples for both nutrient and BOD (biochemical oxygen demand) were taken during 3 consecutive days at 6:45 am, 11:45 am and 4:45 pm. Water from the sump and at the exit of the trickling filters were pooled to improve homogenization using a 40 Liters carboy. This experiment was repeated twice (experiment A and B) with the difference that we included BOD analysis only in experiment B. Logistics problems did not allow as to perform BOD analysis in experiment A.

The volumetric TAN conversion rate (VTR) and Nitrite-N (VNR) conversion rate by the trickling filters were determined using Malone and Pfeiffer's formula [18],

\[
\text{VTR} = \frac{(\text{TANI}-\text{TANE}) \times Qr}{Vb} \\
\text{VNR} = \frac{(\text{Nitrite-NI}-\text{Nitrite-NE}) \times Qr}{Vb}
\]

Where Q, is the flow rate through the filter (m3/d), Vb is the total volume of the media (m3). TANI or Nitrite-NI represents the influent TAN or Nitrite-N concentration (g N m⁻³), and TANE or Nitrite-NE represents the effluent TAN or Nitrite concentration (g N m⁻³).

**Chemical analysis:** Hach reagents for total ammonia nitrogen, TAN (method 8155), nitrate nitrogen (method 8507), nitrite nitrogen (method 8192), TKN (method 8075), Total Kjeldahl Nitrogen refers to the combination of ammonia and organic nitrogen), orthophosphate (method 8048), and total phosphorus; TP (method 8190), were used.

The concentration of each nutrient was determined using a calibration curve in full strength sea water. All readings were recorded on a Hach DR 2000 spectrophotometer. Each value represents an average of four replicates. All samples were filtered through a nominal 0.7 µm pore size, glass fiber filter (Whatman 4.25 cm GF/F Springfield Mills, UK), and hence they were considered as dissolved nutrients.

Several calibrations curves were made using full strength sea water. It was found that the traditional calibration curve approach produced more accurate results than using the recommended program on the spectrophotometer. All calibration was fitted with a blank as zero, and hence they were considered as dissolved nutrients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Slope</th>
<th>SD</th>
<th>CV</th>
<th>Reagents used from HACH Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAN</td>
<td>4</td>
<td>0.569</td>
<td>± 0.010</td>
<td>1.81%</td>
<td>8155</td>
</tr>
<tr>
<td>Nitrite-N</td>
<td>4</td>
<td>0.193</td>
<td>± 0.007</td>
<td>3.7%</td>
<td>8507</td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>9</td>
<td>1.996</td>
<td>± 0.091</td>
<td>4.56%</td>
<td>8192</td>
</tr>
<tr>
<td>TKN-N</td>
<td>4</td>
<td>197.310</td>
<td>± 3.868</td>
<td>1.96%</td>
<td>8075</td>
</tr>
<tr>
<td>Orthophosphate-P</td>
<td>4</td>
<td>0.666</td>
<td>± 0.035</td>
<td>5.28%</td>
<td>8048</td>
</tr>
<tr>
<td>TP-P</td>
<td>4</td>
<td>2.036</td>
<td>± 0.202</td>
<td>9.93%</td>
<td>8190</td>
</tr>
</tbody>
</table>

**Table 1:** This table summarizes value and statistics of each calibration curve (intercept was set as blank or zero) for all parameters analyzed. N represents the number of calibration curves carried out.

**TN/TP=TON/TP**

Nitrogen in the shrimp and fresh feed was determined by the Kjeldahl method. Dry weight or water content was determined by drying at 60°C for 2 days.

**BOD analysis:** BOD was analyzed using weighed corrected BOD bottles (300 mL), covered with aluminum foil and incubated in situ (sump) for 6 hours. Each value represents the average of 5 replicates including the control or time zero. BOD₂₅ was determined by measuring the dissolved oxygen (DO) by the classical Winkler procedure [30]. Water for BOD was collected in a 40 Liters carboy (NaCl) gently aerated. BOD bottles were carefully filled using a rubber tube from the placed at the end of spigot which reach the bottom of the bottles taking care of non-producing air bubbles. The presence or nitrification activity, was carried out by using a nitrifier inhibitor (nitrifier inhibitor formula 2533 (Nitrapyrin) from Hach at 0.6 g bottle⁻¹). Nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] is the most widely used inhibitor of nitrification. It inhibits the first step of nitrification, the oxidation of ammonia to nitrite [31,32].

In order to eliminate any possible interference with nitrite, iron or any reducing or oxidizing compound, 4.5 g L⁻¹ of Sodium azide (Sigma, Co) was included in the Winkler solution [33]. After filling all BOD bottles, they were left for 30 min for acclimation before fixing time zero bottles and or placing the remaining bottles for incubation.

\[
\text{BOD}_{25} = \frac{\text{DOinitial} - \text{DOfinal} \times 24}{\text{Incubation time (h)}}
\]

**Carbon analysis:** The effect of the ratio C:N was studied using 0 (control), 5 and 10 mg CL⁻¹. Water from the sump was placed in 200 Liters fiber glass container with aeration and the corresponding amount of carbon. The organic carbon used was citric acid. BOD was determined in time 0 and every 2 hours. All analysis was carried out using 4 replicates.

**Physic-chemical measurements**

Temperature, pH, dissolve oxygen (DO) and shrimp culture parameters were measured daily in situ. Salinity was measured with a refractometer (Aquatic Ecosystems). Temperature and DO were measured with a Model 550A dissolved oxygen meter (YSI, Yellow Springs, OH). The pH was recorded using an Oaklon pH 5/6 & ion 5/6.

When compared data were analyzed by two-way ANOVA, significant differences between the treatments means were compared by LSD test. Differences were considered significant at an alpha level of 0.05.

**Results**

The average temperature during the time of the experiment was 28.3°C. The pH and oxygen in the sump were always slightly higher than at the drainage of the trickling filters (Table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Temp. oC</th>
<th>pH</th>
<th>O₂ mg L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sump</td>
<td>28.3 ± 0.2</td>
<td>7.7 ± 0.0</td>
<td>6.0 ± 0.0</td>
</tr>
<tr>
<td>Inlet TF</td>
<td>28.3 ± 0.2</td>
<td>7.6 ± 0.0</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>Outlet TF</td>
<td>28.1 ± 0.2</td>
<td>7.5 ± 0.0</td>
<td>5.6 ± 0.3</td>
</tr>
</tbody>
</table>

**Table 2:** Average daily values recorded of temperature °C, pH and oxygen (mg L⁻¹ and % of saturation) in the sump, inlet and outlet of the trickling filter (TF).
of 3,190 broodstock with a total biomass of 123 Kg, equivalent to 3.81 Kg of N. Animals were fed daily with 30 Kg of fresh frozen chopped marine mix which represents a load of 0.462 Kg of N d\(^{-1}\). Broodstock daily mortality was 0.3%. The daily average mating rate was 18%, averting 250,000 and 175,000 eggs and nauplii per female respectively, and with a week difference. Average values are the result of 4 replicates, ± one standard deviation.

Table 4: Daily mass balance of dietary feed dry matter (DM) and Nitrogen (N), where: a waste is defined as the uneaten feed and all excretory compounds (feces and urine).

<table>
<thead>
<tr>
<th>Feed utilization</th>
<th>Nitrogen (g)</th>
<th>% of N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed Input (FI)</td>
<td>462</td>
<td>100</td>
</tr>
<tr>
<td>Waste (^a)</td>
<td>420.9</td>
<td>91.1</td>
</tr>
<tr>
<td>Daily Weight Gain (DWG)</td>
<td>41.1</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Figure 2: TAN and Nitrite-N readings in the sump (S) and after passing the trickling filter (TF) of the Experiments A (above) and B (below). The experiments were carried out during 3 consecutive days at 3 hours, 6:45, 11:45 and 16:45, and with a week difference. Average values are the result of 4 replicates, ± one standard deviation.

TAN and nitrite-N (p<0.05) after passing through the trickling filters (Figure 2). On the other hand, the concentration of nitrate-N did not match the reduction of both TAN and nitrite-N (Figure 3).

The concentration of orthophosphate-P was not affected by the filter treatment (p<0.05). It averaged from 0.436 in the experiment A to 0.376 mg PL\(^{-1}\) in experiment B (Table 5). The average ratio for the ratio N:P was 7.1 for the drainage of the trickling filter to 8.5 in the sump. The dissolved organic nitrogen (DON) and total phosphorus (TP) were more abundant during experiment A than B. DON as percentage of the total nitrogen (TN) ranged from 31.1 to 39.7% in the sump to 37.3 to 43.4% in the discharge of the trickling filter, respectively (Table 6). The volumetric TAN conversion rate (VTR) and Nitrite-N (VNR) conversion rate were 28.6 g N m\(^{-3}\) and 19.9 g N m\(^{-3}\) respectively.

BOD analysis revealed a drop between sump and trickling filter. There was no difference between (p<0.05) samples from the sump incubated with and without the inhibitor Nitrapyrin, in contrast samples taken from the drainage of the trickling filter showed a large variation of results (Table 4).

Discussion

Reports of nitrogen retention in shrimp culture ranges from 23 to 55% and varied with culture condition and species [20,34-37]. Most of the work published has been carried out using dry feed in pond or raceways culture. One exception was the work published by Wickins [38]. Wickins fed fresh feed to *Penaeus monodon* in RAS, and calculated that approximately 80% of the nitrogen of the daily ration...
was not converted into prawn flesh and ended as load on the filters. In the present study only 8.9% of the nitrogen load ended as animal tissue. Shrimp maturation requires fresh frozen feed, and to achieve that uneaten feed and feces represent 79.9% of total nitrogen excretion would represent 12% of the total nitrogen load 64:45:00 0.052 0.002 0.046 0.001 2.709 0.088 0.434 0.014 average 0.055 0.006 0.038 0.001 2.639 0.170 0.436 0.005 stdev 0.014 0.005 0.006 0.001 0.466 0.161 0.029 0.006

Sump

average 0.161 0.009 0.103 0.001 2.877 0.173 0.438 0.006 stdev 0.016 0.005 0.010 0.001 0.323 0.167 0.028 0.003

Trickling filters

average 0.151 0.011 0.106 0.002 2.758 0.088 0.434 0.014

average 0.166 0.005 0.110 0.003 2.814 0.262 0.439 0.006

average 0.148 0.008 0.111 0.002 3.042 0.564 0.461 0.001

average 0.151 0.008 0.106 0.002 3.019 0.263 0.364 0.004
The C:N ratio of the substrates will determine whether or not bacteria take up inorganic nitrogen or will regenerate nitrogen during the mineralization [49-51]. Under the correct conditions bacteria can take up dissolved inorganic nitrogen while simultaneously liberating NH₄ in decomposition [52]. Thus, bacteria can be competing for NH₄, regenerating NH₄, or both. Feeding the system with only high protein diets will result in a low C:N ratio. The C:N ratio of a mean atomic formula of protein, C₁₃₈H₂₁₇O₄₅N₃₉S, is 3.5; this ratio would approach to 4 when considering the fat and carbohydrate content of the fresh feed [53]. In addition, the present study experimentally showed the carbon limitation of the system by measuring the effect of carbon addition on BOD. The oxygen:carbon ratio was 2.86 (Figure 4), very close to the stoichiometric 2.66 found when glucose is oxidized to water and CO₂.

In RAS, fecal bacteria not only inoculate the system but also contribute to its abundance, productivity and process [54]. Interestingly the same authors found an increase in the protein concentration in the feces due partly to bacteria external colonization, a process that eventually will benefit the shrimp through coprophagy [55,56]. Neither the concentration or composition of bacteria was not determined in the present study, however it is unequivocally clear than the semi closed recirculating system described in this study is driven by an attached nitrifiers population restricted to the trickling filters and a general free living or attached heterotrophic population mainly thriving in the sump.

Several problems have been identified and are in process to be studied. It is clear that RAS or closed systems could be reservoirs of pathogenic microorganisms for animal and humans [57], hence horizontal and vertical transmission. There is also a potential accumulation of metabolites, hormones, heavy metals which could impair the rearing embryonic and larval development [58]. A further reduction of water exchange to lower than 10% daily is under study, though the increase in turbidity is an obstacle to face. Turbidity increases the manual work; hence increase stress, hamper the capture of ripped or inseminated females, or generally observing the health of the animals.

The present study shows a simple and inexpensive semi closed recirculating system, running at low C:N ratio, efficient enough to control the concentration of harmful nitrogen. A combination of low C:N ratio and high load of organic nitrogen in the trickling filter could be part of the key factors running this system. This system has supplied to our hatchery an average of 1 billion nauplii per month for the last 8 years. This same concept has been applied with same success in several operations in maturation in other countries in the region.

### References


