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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} d^{-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

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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 m^3 of volume respectively, 258 m^3 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, with a side stream trickling filter, circulation pump (810 L min^{-1} Pacer Inc.), and the maturation tanks. The water returned to the sump by gravity flow (Figure 1A-C).

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 fiber glass towers, each tower with 4 levels of dishes (160 cm of diameter×10 cm of height).

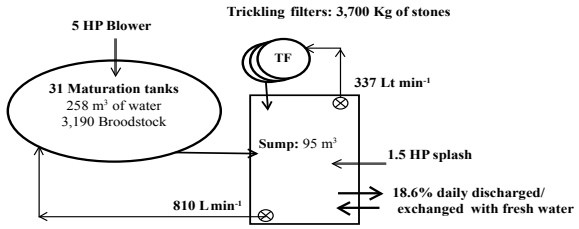

Figure 1a: Diagram of semi closed recirculating system used in Semacua.

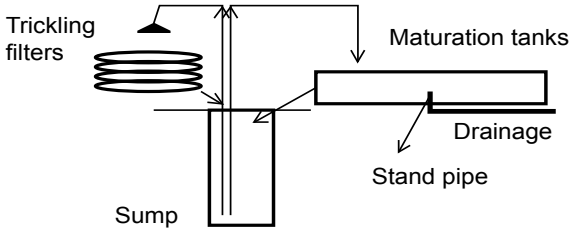

Figure 1b: Side view diagram of the semi closed recirculating system used in Semacua.

Figure 1c: Sump with trickling filters in the semi closed recirculating system used in Semacua.

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), 3700 kg in total. The density and volume of the stones was calculated in 2.58 g mL^{-1} and 1.43 m^3 respectively. Below the bottom dish of each tower, a funnel shaped bottom collect the treated water from the tower and flushes it back to the sump (Figure 1A-C). A 337 L min^{-1} Pacer pump, pulled water from the sump into the battery of trickling filters (Figure 1A-C), and then returned to the sump by gravity. For calculation purposes the water from the sump (95 m^3) passed 5.1 times day^{-1} through the battery of trickling filters.

Aeration was provided in the sump by a 1.5 HP splash Kasco Marine Inc., whereas the maturation tanks were aerated by a 10 HP blower using air stones inside airlifts placed in each tank.

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].

$$VTR\ (g\ N/\ m^3)=(TANI-TAN_E)\times Qr\ /Vb$$

$$VNR\ (g\ N/\ m^3)=(Nitrite\text{-}NI-Nitrite\text{-}N_E)\times Q_r\ /V_b$$

Where Q_r is the flow rate through the filter (m3d-1), Vb is the total volume of the media (m^3). 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), nitrite nitrogen (method 8507), nitrate nitrogen (method 8192), TKN (method 8075, the term TKN, 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 Mill, 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, table 1 shows the slope and statistics for each parameter analyzed. The percentage of recovery was over 90% in all the parameters except in TP that ranged 65%.

Dissolved organic nitrogen (DON) and total nitrogen (TN) were calculated by the following formulas:

DON=TKN-TAN.

TN=TKN+Nitrite-N+Nitrate-N.

The ratio N/P (representing Total inorganic nitrogen (TIN)/soluble reactive phosphate) and TN/TP (TN/TP) was determined as follow:

N/P=TIN/orthophosphate-P

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

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. BOD_{24h} was determined by measuring the dissolved oxygen (DO) by the classical Winkler procedure [30]. Water for BOD was collected in a 40 Liters carboy (Nalgene) slightly 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 gL^{-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.

$$BOD_{24h}=\frac{(DO\,inntial)\text{-}(DO\,final)\times 24}{Incubation\ time\ (h)}$$

Carbon analysis: The effect of the ratio C:N was studied using 0 (control), 5 and 10 $mgCL^{-1}$. 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).

Protein and water content of both fresh feed and broodstock is presented in table 3. The maturation unit consists of a total population

	Temp. oC	pH	O2 mg L^{-1}
Sump	28.3 ± 0.2	7.7 ± 0.0	6.0 ± 0.0
Inlet TF	28.3 ± 0.2	7.6 ± 0.0	5.9 ± 0.3
Outlet TF	28.1 ± 0.2	7.5 ± 0.0	5.6 ± 0.3

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).

Fresh feed		Broodstock	
% N	% water	% N	% water
1.54	84.41	3.10	72.98
± 0.12	± 0.31	± 0.10	± 0.26

Table 3: Nitrogen and water content of the frozen feed and Broodstock (± one standard deviation). Average values are the result of 4 replicates, ± one standard deviation.

Feed utilization	Nitrogen (g)	% of N
Feed Input (FI)	462	100
Waste [a]	420.9	91.1
Daily Weight Gain (DWG)	41.1	8.9

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).

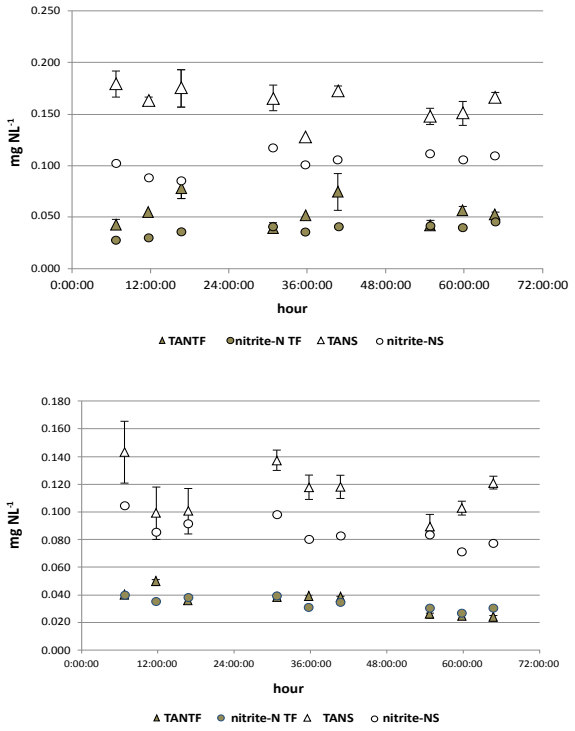

Figure 2: TAN and Nitrite-N readings in the sump (S) and after passing the trickling filter (TF) of the Experiments A (above) and B (below). The experiments were carried out during 3 consecutive days at 3 hours, 6:45, 11:45 and 16:45, and with a week difference. Average values are the result of 4 replicates,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

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%, averaging 250,000 and 175,000 eggs and nauplii per female respectively, equivalent to a 70% hatch rate. Average growth rate of non-mature female and male was 1.5 and 1.4 g $week^{-1}$ respectively. GSI of mating population was 6.43 ± 0.7%. Daily nitrogen retention was calculated at 41.1 g d^{-1} which is equivalent to 8.9% of the total nitrogen load.

A deduced daily mass balance is presented in table 4. This table presents the nitrogen retention (8.9%) by broodstock and the waste, as the sum of non-eaten feed, and excretion.

Dissolved inorganic nutrients for experiments A and B are shown in tables 5 and 6 and figures 2 and 3. There is a clear reduction of both 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

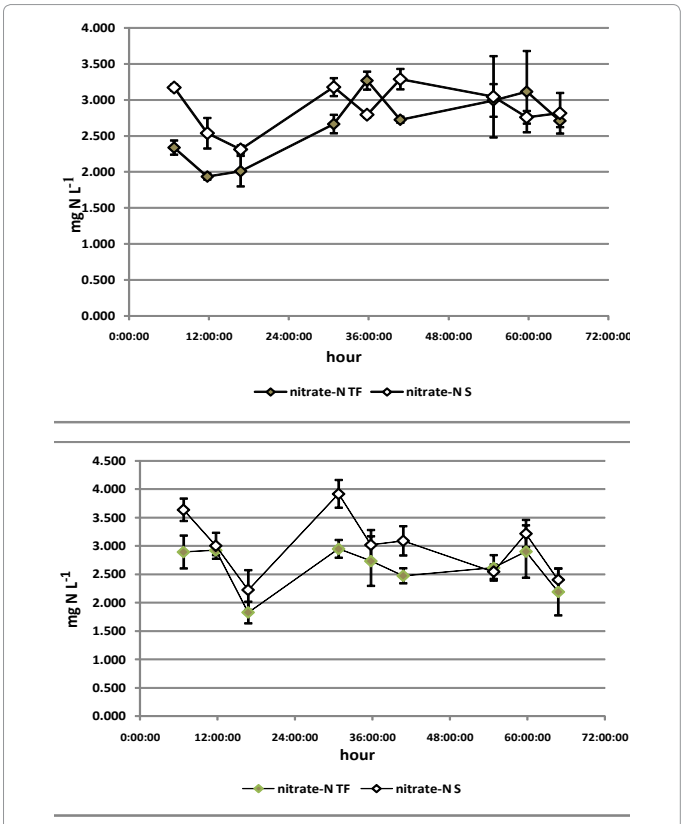

Figure 3: Nitrate-N readings in the sump (S) and after passing the trickling filter (TF) of the Experiments A (above) and B (below). The experiments were carried out during 3 consecutive days at 3 hours, 6:45, 11:45 and 16:45, and with a week difference. Average values are the result of 4 replicates,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

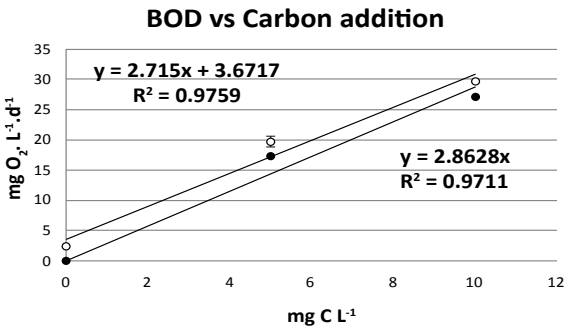

Figure 4: Relationship between BOD and organic carbon. Open circles are actual measured values. In order to determine the concentration of O_2 needed to oxidize 1 mg of carbon, closed circles representing zeroed control were included. Each point represents the average of 4 replicates ± one standard deviation.

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 a good maturation, broodstock demands to be fed *ad libitum* or in excess. Calculating the amount of the wasted feed is extremely hard, but it would not be wrong to suggest that most of the waste (91.9%) were uneaten feed, and it can be partly attributed to the nibbling habit of crustaceans [39,40].

It is known that all aquatic crustaceans are strictly ammonotelic [41], and the rate of excretion is related to salinity [42], as well as size, concentration of TAN, molt stage, feed intake, temperature, salinity, pH and dissolved oxygen [43]. By assuming an excretion rate of 13.11 µg. TANgh^{-1} [38,43,44], and considering that TAN represents 70% of the nitrogen excreted , the total nitrogen and TAN excretion nitrogen rate for this study would be 55.3 and 39.7 g N d^{-1} respectively [43]. Thus, the total nitrogen excretion would represent 12% of the total nitrogen load (462 g N d^{-1}), resulting that uneaten feed and feces represent 79.9% of the nitrogen load, a percentage close to reported by Wickins [38].

Both TAN and Nitrite-N were rapidly nitrified in the trickling filters (Table 5 and Figure 2), interestingly, the Nitrate-N fraction did not increase accordingly to expectations (Table 5 and Figure 3). Nitrate-N concentration was kept almost similar with a slightly tendency to decrease after passing through the trickling filters (Figure 3). It is tempting to suggest that concentration of Nitrate-N was the product of nitrification and in a small degree denitrification within the trickling filters (Table 5).

The trickling filter is a biofilm fed by a diffusion controlled process driven by concentration gradient across the film; both physical and chemical conditions influence the filter performance [17,45]. A decrease in oxygen to below suboptimal levels from the external boundary to within the biofilm is not uncommon. An increase in organic or TAN loading will cause more consumption of oxygen [46]. Schramm and coworkers working in a trickling filter of an eel water recirculation system found that nitrification was restricted to a narrow zone of 50 microns on the very top of the film [47]. Similarly to the hyporheic zone, trickling filters will create a variety of microniches, promoting the activity of some otherwise poor competitors, such as chemolithotrophers, especially if low dissolved carbon is present. A variety of anaerobic respiratory pathways, such as nitrate, ferric ion, sulphate and even methanogenic respiration will be employed even in aerobic sediments [48]. Figure 3 shows an almost constant drop of Nitrate-N at the drainage of the trickling filter, more likely as result of low oxygen to anaerobic pockets in the trickling filter. These pockets could be the result of the constant organic load; its development will depend on both, the amount of organic load and transit time. In this connection, BOD from water passed through the trickling filters was lower than water from the sump (Table 6), demonstrating the filtration capabilities of the trickling filters and hence the drop of oxygen and pH shown in table 2.

Both Nitrate-N and DON accounted for about 95% of the dissolved nitrogen pool in the system. It is obvious that the concentration of both is the result of bioaccumulation.

The lack of effect of the nitrifying inhibitor in water taken from the sump, suggest that there are two different processes occurring in

Exper. A	TAN		Nitrite-N		Nitrate-N		orthophosphate-P	
Trickling filters								
Time	av	±	av	±	av	±	av	±
6:45:00	0.043	0.005	0.028	0.000	2.338	0.098	0.400	0.002
11:45:00	0.055	0.001	0.030	0.001	1.936	0.043	0.411	0.002
16:45:00	0.078	0.010	0.036	0.000	2.010	0.212	0.422	0.016
30:45:00	0.040	0.004	0.041	0.001	2.664	0.128	0.470	0.004
35:45:00	0.052	0.003	0.036	0.002	3.267	0.125	0.405	0.001
40:45:00	0.075	0.018	0.040	0.000	2.724	0.043	0.437	0.003
54:45:00	0.042	0.004	0.042	0.001	2.992	0.227	0.482	0.004
59:45:00	0.057	0.004	0.040	0.000	3.114	0.564	0.439	0.001
64:45:00	0.052	0.002	0.046	0.003	2.709	0.088	0.455	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								
6:45:00	0.179	0.012	0.102	0.000	3.171	0.043	0.469	0.002
11:45:00	0.162	0.004	0.088	0.001	2.536	0.212	0.392	0.016
16:45:00	0.175	0.019	0.085	0.003	2.310	0.057	0.423	0.006
30:45:00	0.165	0.012	0.117	0.001	3.177	0.125	0.468	0.001
35:45:00	0.127	0.003	0.101	0.002	2.794	0.043	0.401	0.003
40:45:00	0.172	0.005	0.106	0.002	3.287	0.142	0.456	0.002
54:45:00	0.148	0.008	0.111	0.000	3.042	0.564	0.461	0.001
59:45:00	0.151	0.011	0.106	0.002	2.758	0.088	0.434	0.014
64:45:00	0.166	0.005	0.110	0.003	2.814	0.282	0.439	0.006
Trickling filters								
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	0.006
6:45:00	0.040	0.003	0.040	0.000	2.894	0.289	0.425	0.003
11:45:00	0.050	0.008	0.035	0.001	2.924	0.156	0.391	0.008
16:45:00	0.036	0.002	0.039	0.001	1.828	0.224	0.341	0.003
30:45:00	0.039	0.005	0.039	0.001	2.950	0.086	0.427	0.006
35:45:00	0.039	0.007	0.031	0.000	2.734	0.437	0.366	0.002
40:45:00	0.038	0.008	0.035	0.000	2.475	0.461	0.365	0.006
54:45:00	0.026	0.007	0.030	0.001	2.614	0.191	0.390	0.009
59:45:00	0.024	0.002	0.027	0.001	2.902	0.133	0.351	0.007
64:45:00	0.024	0.006	0.030	0.001	2.190	0.413	0.346	0.012
average	0.035	0.005	0.034	0.001	2.612	0.266	0.378	0.006
stdev	0.009	0.002	0.005	0.000	0.388	0.141	0.032	0.003
Sump								
6:45:00	0.143	0.022	0.105	0.001	3.638	0.197	0.430	0.020
11:45:00	0.099	0.007	0.085	0.002	3.004	0.244	0.388	0.002
16:45:00	0.100	0.009	0.091	0.001	2.224	0.127	0.337	0.003
30:45:00	0.137	0.019	0.098	0.001	3.919	0.229	0.419	0.003
35:45:00	0.118	0.009	0.080	0.001	3.019	0.263	0.364	0.004

Table 5: Dissolved inorganic nutrients recorded in Experiment A (above) and B (below). All data expressed as mg L^{-1}. Average values are the result of 4 replicates, ± one standard deviation.

Exp. A	TKN-N		DON	TN	TP		TN/TP
Trickling filters							
Time	av	±	av	±	av	±	av
6:45	2.269	0.977	2.226	4.634	0.416	0.004	11.131
11:45	2.466	0.140	2.412	4.433	0.426	0.006	10.417
16:45	3.453	0.698	3.375	5.499	0.454	0.012	12.112
6:45	2.269	0.419	2.229	4.974	0.461	0.004	10.785
11:45	1.677	0.698	1.625	4.981	0.454	0.012	10.970
16:45	2.466	0.698	2.392	5.231	0.462	0.020	11.318
6:45	1.677	0.140	1.635	4.711	0.527	0.003	8.934
11:45	1.283	0.419	1.226	4.436	0.450	0.003	9.859
16:45	1.776	1.116	1.724	4.530	0.485	0.003	9.349
average	2.149	0.589	0.589	4.825	0.459	0.007	10.542
stdev	0.639	0.340	0.631	0.372	0.032	0.006	1.009
Sump							
6:45	2.960	0.140	2.781	6.233	0.520	0.006	11.982
11:45	1.579	0.698	1.416	4.203	0.422	0.012	9.949
16:45	2.368	1.395	2.193	4.763	0.432	0.013	11.035
6:45	2.171	0.419	2.023	5.324	0.499	0.003	10.674
11:45	1.184	1.116	1.033	4.048	0.458	0.003	8.837
16:45	2.368	1.116	2.202	5.292	0.462	0.009	11.450
average	2.236	0.791	2.076	5.216	0.471	0.009	11.051
stdev	0.560	0.382	0.559	0.736	0.034	0.005	1.134
Trickling Filters							

Exper. B	TKN-N	DON	TN		Tp		TN/TP	BOD	
Time	av	±	av	±	av	±	av	±	av
6:45	2.171	.395	2.131	5.105	0.479	0.011	10.658	0.556	0.468
11:45	0.839	0.519	0.789	3.798	0.357	.009	10.628	0.973	0.543
16:45	1.924	0.691	1.888	3.790	0.438	0.003	8.648	0.703	0.592
6:45	1.677	0.613	1.651	4.322	0.420	0.011	10.280	0.541	0.325
11:45	1.924	0.567	1.900	4.853	0.415	0.010	11.699	0.717	0.116
16:45	1.924	0.888	1.900	4.144	0.392	0.013	10.561	1.231	0.454
average	1.628	0.591	1.593	4.274	0.417	0.009	10.289	0.896	0.392
stdev	0.479	0.149	0.484	0.543	0.039	0.004	1.257	0.269	0.150
6:45	1.332	0.296	1.189	5.074	0.474	0.007	10.697	1.311	0.476
11:45	0.789	0.426	0.690	3.878	0.358	0.004	10.822	1.437	0.459
16:45	2.318	0.672	2.218	4.634	0.417	0.013	11.102	1.434	0.305
6:45	2.417	0.652	2.328	5.047	0.411	0.004	12.287	1.556	0.362
11:45	1.332	0.589	1.214	4.431	0.377	0.003	11.747	1.145	0.490
16:45	1.283	0.342	1.165	4.456	0.396	0.013	11.253	1.280	0.221
6:45	2.417	0.652	2.328	5.047	0.411	0.004	12.287	1.556	0.362
11:45	1.381	0.426	1.279	4.670	0.423	0.011	11.040	0.875	0.408
16:45	1.727	0.888	1.606	4.207	0.382	0.005	11.019	1.304	0.168
average	1.557	0.575	1.442	4.649	0.411	0.007	11.300	1.304	0.341
stdev	0.520	0.220	0.527	0.481	0.039	0.004	0.518	0.199	0.127

Table 6: Dissolved organic nutrients N/P ratio and BOD recorded in Experiment A (above) and B (below). All data expressed as mg L^{-1} or mg L^{-1} d^{-1} in BOD. Average values are the result of 4 replicates, ± one standard deviation.

our recirculation system. On one hand, the trickling filter with a major nitrifying activity and a heterotrophic counterpart present in both the sump and rearing tanks.

As general rule, an estimated of the TAN generated per day in an aquaculture-heterotrophic production can be calculated using the following equation:

P_{TAN}=F×PC×0.144; where P_{TAN} is the production rate of TAN (Kg/day); F is the feed rate and PC the protein concentration of the feed (decimal value) [16]. The application of this equation in the study would result in a daily production of 66.5 g of TAN, than divided by the total volume of the system would represent about 0.18 mg TAN L^{-1}, concentration similar to found in the system in the present study.

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 NH_4 in decomposition [52]. Thus, bacteria can be competing for NH_4, regenerating NH_4, 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, $C_{138}H_{217}O_{45}N_{39}S$, 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 CO_2.

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 than 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 to 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.

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