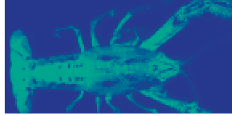
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Nitrogen budget for a low-salinity, zero-water exchange culture system: II. Evaluation of isonitrogenous feeding of various dietary protein levels to *Litopenaeus vannamei* (Boone)

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Abstract

This study evaluated the effects of isonitrogenous feeding (60 g dietary protein per kilogram of body weight per day) using experimental feeds with 25%, 30%, 35% and 40% protein on the nitrogen budget, ammonia efflux rate, growth and survival of juvenile *Litopenaeus vannamei* raised in a low-salinity ($4\,g\,L^{-1}$) zero-water exchange culture system for 4 weeks. No significant differences in weight gain or instantaneous growth rate were observed between the dietary treatments with 35% and 40% protein after 3 weeks of study, or between treatments with 25% and 30% protein after 4 weeks of study. High mortality rates were observed for the 35% and 40% protein treatments, probably associated with high nitrite levels (4.80 and 7.36 mg NO_2-NL^{-1} respectively) in water. Among the various dietary treatments, 39–46.3% of feed nitrogen was converted to shrimp biomass, 32.8–38.0% and 14.4–39.9% remained within the system as organic and inorganic nitrogen, respectively, and 32.5–39.3% was unaccounted for. The results of the present study showed high nitrogen utilization efficiencies. However, as the nitrogen loading of the zero-water exchange system increased, so did the nitrogen excretion of shrimp, causing a deteriorated general condition of the shrimp, demonstrated by the low ammonia efflux rates recorded at the end of the trial. This study confirms that low-salinity closed systems are particularly susceptible to nitrogen loading. Thus, in these culture systems, low-protein feeds may perform better as they provide more carbon for heterotrophic bacteria and less nitrogen to be degraded and transformed into nitrogenous wastes.

Keywords: shrimp, zero-water exchange, low salinity, nitrogen budget, isonitrogenous feeding, ammonia efflux rate

Introduction

Increasing concerns over the environmental impact of shrimp farming and the incidence of disease have led to a progressive reduction in water exchange up to the development of nearly static systems with no water exchange, where effluent discharge and the introduction and dissemination of pathogens are significantly reduced (Burford, Thompson, McIntosh, Bauman & Pearson 2003). Moreover, outdoor culture systems with no water exchange have been proven to be beneficial for shrimp culture as higher growth and final weights have been attained with the Pacific white shrimp, *Litopenaeus vannamei* (Boone) (Browdy & Bratvold 2001; Tacon, Cody, Conquest, Divakaran, Forster & Decamp 2002). These favourable results have been explained by the large availability of food

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organisms such as bacteria, phytoplankton, zooplankton and nematodes that develop within the system by reutilizing dissolved nutrients from uneaten feed, faeces and nitrogenous wastes (Burford, Preston, Glibert & Dennison 2002). They can be used as additional food sources by shrimp (Tacon *et al.* 2002; Decamp, Cody, Conquest, Delanoy & Tacon 2003) and may allow a reduction in the protein content and other nutrients in balanced feeds (Tacon *et al.* 2002). Thus, the establishment of the protein requirement of shrimp raised in static systems is of great interest as protein is the most expensive dietary component and a key limiting nutrient for shrimp growth (Kureshy & Davis 2002).

Studies on dietary protein requirement for *L. vannamei* suggest that values range from 15% to 36% (Smith, Lee, Lawrence & Strawn 1985; Cousin, Cuzon, Blanchet & Ruelle 1991; Aranyakananda & Lawrence 1993; Cruz-Suárez, Antonio-Pérez, Luna-Mendoza, Tapia-Salazar, Guajardo-Darbosa & Ricque-Marie 2000; Velasco, Lawrence, Castille & Obaldo 2000). However, the protein requirement varies with respect to changes in biotic (shrimp species, size) and abiotic factors (temperature, salinity), which partly explains the different values. Therefore, the protein requirement has been defined more adequately as the amount of protein required per animal biomass per day (Kureshy & Davis 2002), and it is often based on the response to varying levels of dietary protein under a particular set of conditions that would produce the most favourable production response, i.e. weight gain, feed efficiency or protein conversion efficiency. However, the protein requirement can be met with balanced feeds of different dietary protein content, given that a low level of dietary protein, for example, could be compensated for by higher ingestion or vice versa. Consequently, one could provide an equal amount of protein and nitrogen with diets of different dietary protein contents by applying isonitrogenous feeding.

An additional relevant aspect of dietary protein is its role as a potential pollutant. In theory, within static systems, nitrogen metabolites tend to accumulate over time, including toxic forms such as ammonia and nitrites; however, it is evident that shrimp are capable of living and growing just as well, or better, in these systems as in open systems. As nitrogen is closely associated with protein, the study of its distribution and dynamics by means of a nitrogen budget provides information not only on assimilation efficiency and transformation into shrimp biomass, but at the same time, it constitutes an evaluation of the culture system (Thoman, Ingall, Davis & Arnold 2001). A nitrogen budget of a static system could provide important information on the relationship between the biological performance of shrimp and the concentration of nitrogenous metabolites. As the ammonia concentration in culture systems increases, the ammonia excretion rate of shrimp decreases as levels in the haemolymph and tissues also increase. Under these circumstances, a reduction in the growth rate is commonly observed (Colt & Armstrong 1981). In view of these considerations, the objectives of this study were to evaluate the effect of isonitrogenous feeding of various dietary protein levels to *L. vannamei* cultured in a low-salinity zero-water exchange system, develop a nitrogen budget and determine ammonia efflux rates.

Materials and methods

Experimental system

A 4-week growth trial was conducted in indoor aquaria (bottom area 0.23 m^2) filled with diluted seawater of 4 $g\,L^{-1}$. Four experimental diets with 25%, 30%, 35% and 40% protein were randomly assigned to five aquaria each. An additional aquarium (bottom area 0.49 m^2) per dietary treatment was maintained under the same experimental conditions and with the same stocking density in order to keep shrimp for the evaluation of ammonia excretion rates. The feeding rate (Table 1) was adjusted to provide an isonitrogenous feeding, i.e., an equal amount of dietary protein (DP) per kg of body weight (BW) per day (d), which was 60 $g\,DP\,kg\,BW^{-1}d^{-1}$, a common feeding rate for commercial feeds containing 40% dietary protein, and also similar to the protein requirement for maximum growth reported by Kureshy and Davis (2002) for this species and size. Daily measurements of temperature and salinity

Table 1 Feeding rates for the isonitrogenous feeding of juvenile *L. vannamei*using experimental diets containing 25%, 30%, 35% and 40% dietary protein

Treatment (% protein)	Feeding rate (% BW day^{-1})	Protein provided ($g\,DP\,kg\,BW^{-1}\,day^{-1}$)
25	24	60
30	20	60
35	17	60
40	15	60

BW, wet body weight; DP, dietary protein.

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were taken; the mean values ($\pm$ SD) were 30.1 $\pm$ 0.5 °C and 4.0 $\pm$ 0.1 g L^{-1} respectively. Dissolved oxygen was also monitored daily and averaged 6.4 $\pm$ 0.6 mg L^{-1}; pH was monitored twice a week and averaged 8.5 $\pm$ 0.1. No water exchange was performed but freshwater, dechlorinated by vigorous overnight aeration, was added once a week to replace water lost due to evaporation.

Experimental diets

The practical diets containing 25%, 30%, 35% and 40% protein were synthesized at the laboratory of Auburn University, Department of Fisheries and Allied Aquacultures. The ingredients of the basal composition were the same as described previously (González-Félix, Gómez-Jiménez, Perez-Velazquez, Davis & Velazco-Rameños 2007). The dry ingredients were mixed in a mixer (Hobart, Troy, OH, USA) for 30 min, after which the lipids were blended in and hot water was added until the appropriate consistency for pelleting was obtained. The diets were then passed through a meat grinder using a 2 mm die. The pellets were dried ($<$45 °C) in a forced-air oven to a moisture content of $<$10%. All diets were stored at $-$30 °C until the beginning of the experimental trial, when they were mechanically crumbled and sieved to the desired size. The daily ration was fed to shrimp 15 times a day using automatic feeders. At the end of the trial, triplicate samples of each diet were used for protein analysis.

Experimental shrimp

Litopenaeus vannamei postlarvae were obtained from Maricultura del Pacífico, S.A. de C.V. (Bahía de Kino, Sonora, Mexico). They were acclimated to laboratory conditions at the Laboratory of Bioassays of the Centro de Investigación en Alimentación y Desarrollo (C.I.A.D., A.C., Hermosillo, Sonora, Mexico) at 28.5 °C and 36 g L^{-1}. Once the animals reached an individual weight of approximately 0.3 g, they followed an acclimation procedure to a salinity of 4 g L^{-1} described previously (González-Félix *et al.* 2007). Shrimp were fed a commercial postlarval feed (Camaronina, Agribrands Purina^{MR}, Ciudad Obregón, Sonora, Mexico) with 40% dietary protein until the beginning of the trial. Ten shrimp of similar size were blotted dry, weighed and stocked into the aquaria at a density of 43 shrimp m^{-2}. To maintain a constant stocking density and feeding rate throughout the experiment, dead animals in each aquarium were substituted with marked shrimp of similar size, maintained under similar experimental conditions in 4 g L^{-1} water. The mark consisted of a polyester colour band tied around the eyestalk. At the end of the feeding trial, shrimp with no marks were weighed as a group. The initial and final weights were calculated by dividing the group weight by the number of shrimp weighed. After termination of the experiment, shrimp were stored at $-$80 °C.

Nitrogen analyses and budget

Two initial shrimp samples consisting of 10 individuals each, and three shrimp samples per treatment at the end of the trial (each consisting of tissue from three whole shrimp) were thawed and dried for 24 h at 75 °C, and then weighed and macerated to obtain a fine dust. Nitrogen quantification was carried out in duplicate for each feed and shrimp sample using Microkjeldahl analyses (Official Method 990.03, AOAC 2000). Weekly samples were collected for inorganic nitrogen, determined as total ammonia nitrogen (NH_4^+-N), nitrite (NO_2-N), and nitrate (NO_3-N), as well as for dissolved organic nitrogen (DON), suspended solids nitrogen (SSN), settable solids nitrogen (SetSN), the concentration of chlorophyll *a* for the estimation of phytoplankton biomass and for the estimation of bacterial growth. All procedures concerning collection and analysis of samples were the same as described previously (González-Félix *et al.* 2007).

The total nitrogen input (TNI) per aquarium was determined at the end of the trial as

$$\text{TNI per aquarium} = \text{Total amount of feed given per aquarium} \times \text{Mean dry matter content of the feed} \times \text{Fraction of nitrogen contained in dry feed}$$

Thus, the TNI was considered to be 100% of the nitrogen introduced, and ought to be equivalent to the total nitrogen recovered (TNR) per aquarium, expressed as

$$\text{TNR per aquarium} = N_{SHRIMP} + N_{TON} + N_{TIN} - N_{INITIAL}$$

N_{SHRIMP} indicates the total amount of nitrogen incorporated into shrimp biomass at the end of

the trial per aquarium. N_{TON} is the total amount of organic nitrogen present per aquarium, including DON, SSN and SetSN. N_{TIN} is the total amount of inorganic nitrogen present per aquarium, including ammonia, nitrite and nitrate. All organic and inorganic nitrogen subtracted during the trial in water samples, as well as in dead shrimp removed from each aquarium were determined and incorporated back into N_{TON}, N_{TIN} and N_{SHRIMP} respectively. Finally, $N_{INITIAL}$ considered all nitrogen present at the beginning of the trial in newly stocked shrimp, as well as all organic and inorganic nitrogen in the water for each and every aquarium, which was then deducted from the TNR in order to establish the nitrogen balance. The nitrogen deficit or the total nitrogen unaccounted per aquarium (TNUA) was determined as

$$\mathrm{TNUA} = \mathrm{TNI} - \mathrm{TNR} \quad \text{or} \quad \%\mathrm{TNUA} = 100 - \%\mathrm{TNR}$$

Ammonia efflux rate

Ammonia efflux rates were determined for shrimp at the beginning of the experiment, before and after the acclimation to $4\,g\,L^{-1}$, and at the end of the third and fourth week of the trial. At initiation, ammonia efflux rates from random samples of five starved shrimp kept unfed for 24 h in seawater of $36\,g\,L^{-1}$, and five unfed animals maintained at $4\,g\,L^{-1}$ were measured. Each shrimp was stocked into individual 1 L round glass aquaria filled with 500 mL of clean filtered 4 or $36\,g\,L^{-1}$ water at 28.5 °C. After following the procedures described previously (González-Félix *et al.* 2007), weight-specific ammonia efflux rates were calculated considering individual shrimp weight, the time between sample collections and the water volume. Total ammonia, which refers to the sum of NH_3 and NH_4^+, was quantified using a flow injection/gas diffusion (FIA) technique.

Statistical analysis

Final weight, instantaneous growth rate (IGR) and survival were the indices used to evaluate shrimp performance. Instantaneous growth rate was calculated from the equation IGR = 100 × [ln (Final weight/Initial weight)]/duration of feeding trial in days (Cushing 1968). Survival was transformed by arcsine square root before statistical analysis. These data, as well as weekly observations of ammonia, nitrite, nitrate, DON and SSN, and the various components of the nitrogen budget at the end of the trial, were analysed using one-way analysis of variance (ANOVA) to determine significant ($P < 0.05$) differences among treatment means. Duncan's multiple range test was used as the mean separation procedure. Statistical analyses were performed using the SAS software package (SAS Institute Inc. 1999–2000).

Results

Biological performance of shrimp

No significant differences among dietary treatments were observed for initial weight at the beginning of the experiment. Owing to low survival rates observed for the dietary treatments with 35% and 40% protein, they were terminated after 3 weeks in order to have enough animals for tissue analysis. The treatments with 25% and 30% dietary protein were kept for another week. Therefore, statistical comparisons were performed between treatments with 35% and 40% protein on week 3, with no significant differences detected for final weight, weight gain or IGR. Likewise, no significant differences were detected between treatments with 25% and 30% protein on week 4 for those same variables (Table 2). With respect to survival, data were collected for all four treatments on week 3. Treatments with 25% and 30% protein showed significantly higher survival than treatments with 35% and 40% protein (Table 2). After 4 weeks, no statistical differences were observed between survival of shrimp fed diets with 35% and 40% protein (Table 2).

Nitrogen metabolites and budget

Statistical comparisons for nitrogen metabolites were made between all dietary treatments up to week 3, and between treatments with 25% and 30% protein on week 4. The highest ammonia concentration ($2.65\,mg\,NH_4\text{-}N\,L^{-1}$) was recorded after 4 weeks for the treatment with 25% of dietary protein (Fig. 1), and its overall mean concentration ($0.57\,mg\,NH_4\text{-}N\,L^{-1}$) was significantly higher than those of the rest of the dietary treatments (0.12, 0.12 and 0.05 mg $NH_4\text{-}N\,L^{-1}$ for the 30%, 35% and 40% protein treatments respectively). Nitrite concentration showed significant differences among the dietary treatments during the first 3 weeks of the trial; the highest concentrations were recorded for the 40% and 35% protein treatments on the third week (7.36 and

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Table 2 Initial and final weight, weight gain, instantaneous growth rate (IGR) and survival of juvenile *L. vannamei* after 3* and 4** weeks of isonitrogenous feeding with different dietary protein levels in a low-salinity zero-water exchange culture system*

Treatment (% dietary protein)	Initial weight (g)	Final weight (g)	Weight gain (g) †	IGR (% d^{-1})	Survival (%)
25	0.35 ± 0.01	1.96 ± 0.21**	1.61 ± 0.21**	6.12 ± 0.42**	85.4 ± 9.2a*
					63.0 ± 31.2**
30	0.36 ± 0.02	2.09 ± 0.17**	1.73 ± 0.16**	6.28 ± 0.14**	90.0 ± 0.0a*
					78.8 ± 13.5**
35	0.35 ± 0.02	1.13 ± 0.38*	0.78 ± 0.36*	6.08 ± 1.55*	35.8 ± 24.2b*
40	0.36 ± 0.01	1.21 ± 0.08*	0.85 ± 0.07*	6.42 ± 0.16*	48.1 ± 30.9b*

*Values are means of five replicates ± SD. Means within columns with the same letter and for the same week are not significantly different ($P<0.05$).

†Weight gain = final weight – initial weight.

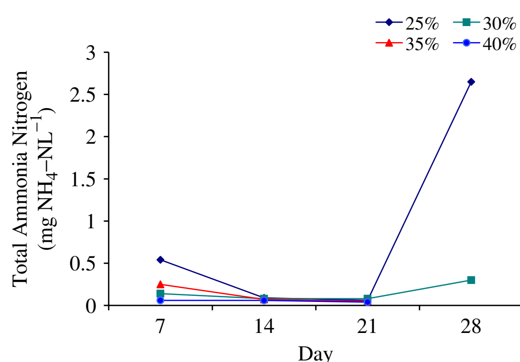

Figure 1 Total ammonia nitrogen (mg NH_4-N L^{-1}) during a 4-week growth trial evaluating four dietary protein levels fed on an isonitrogenous-feeding regime to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent means of five replicate observations.

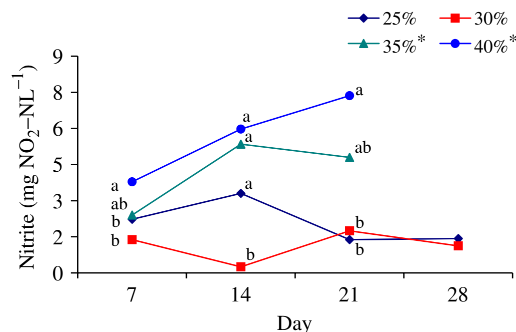

Figure 2 Total nitrite (mg NO_2-N L^{-1}) during a 4-week growth trial evaluating four dietary protein levels fed on an isonitrogenous-feeding regime to *Litopenaeus. vannamei* raised in a low-salinity zero-water exchange culture system. Values represent means of five replicate observations. Means within weeks with the same letter are not significantly different ($P<0.05$).

4.80 mg NO_2-N L^{-1}, respectively. Fig. 2). Nitrate concentration was significantly higher for the 40% protein treatment during the first and the third week of this trial (9.47 and 13.32 mg NO_3-N L^{-1}, respectively. Fig. 3).

The highest DON value was found during the third week for the 40% protein treatment (8.60 mg NO_3-N L^{-1}, Fig. 4) and it was significantly higher than the values recorded for the 25% and 30% protein treatments. SSN showed the highest concentrations in the treatment with 35 (1.86 and 1.88 mg NO_3-N L^{-1} on days 14 and 21 respectively) and 40% dietary protein (1.72 mg NO_3-N L^{-1} on day 14, Fig. 5). SetSN showed differences among treatments after 3 weeks; the concentration of the 25% protein treatment was significantly higher (3.87 mg NO_3-N L^{-1}) than those of the other treatments (1.42, 2.12 and 2.01 mg NO_3-N L^{-1} for the 30%, 35% and 40% protein treatments respectively). By the end of this trial, the concentration had increased to 4.76 and 3.51 mg NO_3-N L^{-1} in the 25% and 30% protein treatments.

Nitrogen (11.2%) and protein (70.0%) contents for initial shrimp and final shrimp were determined at the end of the trial. The protein content for animals fed 25%, 30%, 35% and 40% of dietary protein was 71.6%, 71.9%, 71.7% and 72.2%, respectively, expressed on a dry-weight basis. Analysis of the experimental diets showed a protein content of 27.6%, 32.3%, 37.0% and 42.0% for the 25%, 30%, 35% and 40% protein diets respectively.

Chlorophyll *a* level at the beginning of this trial was 0.0013 μg L^{-1}. After termination of the trial, it averaged 0.0087, 0.0057, 0.0089 and 0.0107 μg L^{-1} for the 25%, 30%, 35% and 40% protein treatments, respectively, indicating a very low phytoplankton

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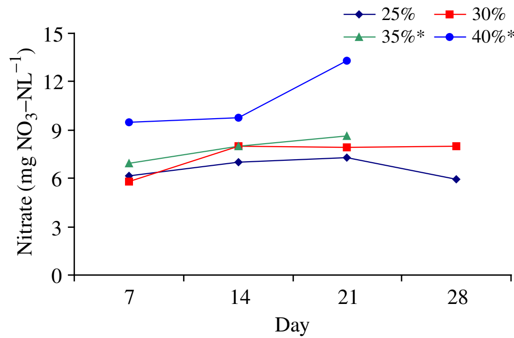

Figure 3 Total nitrate (mg NO_3-N L^{-1}) during a 4-week growth trial evaluating four dietary protein levels fed on an isonitrogenous-feeding regime to *Litopenaeus annamei* raised in a low-salinity zero-water exchange culture system. Values represent means of five replicate observations.

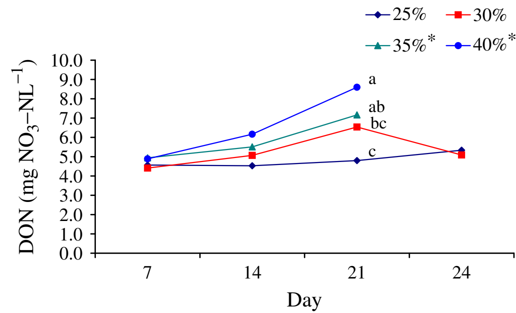

Figure 4 Dissolved organic nitrogen (DON, mg NO_3-N L^{-1}) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent means of five replicate observations.

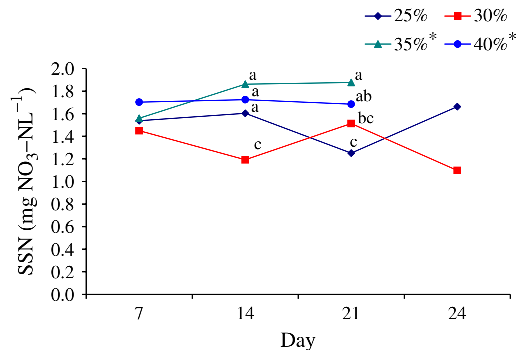

Figure 5 Suspended solids nitrogen (SSN, mg NO_3-N L^{-1}) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent means of five replicate observations.

development. Thus, nitrogen uptake by phytoplankton was considered to be negligible. An estimate of bacterial degradation of organic nitrogen and bacterial assimilation of inorganic nitrogen was not accomplished as it was not possible to quantify bacterial surface growth accumulation on the microscope slides with an electronic scale accurate to four decimal places (Mettler AE240, Hightstown, NJ, USA).

After 3 weeks of culture, no significant differences were detected between the treatments with 35% and 40% protein for the various components of the nitrogen balance (Table 3). After four weeks, the incorporation of nitrogen into shrimp biomass for the treatment with 30% protein was significantly higher than for the treatment with 25% protein. No statistical differences were detected between these treatments for N_{TON}, N_{TIN} or TNUA (Table 3).

Ammonia efflux rates

The initial (control) ammonia efflux rates of juvenile *L. vannamei* before and after acclimation to low salinity (36 and 4 g L^{-1} respectively) are shown in Fig. 6. Both groups showed the highest value after the first hour following transfer of the animals to individual experimental aquaria, possibly reflecting a handling effect. These values were not included, therefore, in the calculation of the mean ammonia excretion rate of 3.19 ± 0.41 and 2.03 ± 0.50 μmol NH_4-N g^{-1} h^{-1} for 4 and 36 g L^{-1} respectively. After 3 weeks of exposure to culture conditions, the lowest efflux rates were recorded for shrimp fed the 30% and 25% protein diets and the highest were measured in shrimp fed the 35% and 40% protein diets (Fig. 7). As a result of mortality, after 4 weeks of exposure, we were able to measure the ammonia efflux rates only for the shrimp fed the 25% and 30% protein diets; after 9 h, low efflux rates in shrimp were recorded from both treatments: 0.76 ± 0.04 and 0.61 ± 0.04 μmol NH_4-N g^{-1} h^{-1} for the 25% and 30% protein treatments respectively (Fig. 8).

Discussion

During this trial, water quality parameters such as temperature, dissolved oxygen and pH remained at acceptable levels for this species. Final weight and IGR showed that using an isonitrogenous-feeding

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Table 3 Nitrogen budget for a low-salinity zero-water exchange culture system where *L. vannamei* was raised for 3* and 4** weeks on an isonitrogenous feeding regime with four dietary protein levels (25%, 30%, 35% and 40%)*

Dietary treatment (%)	Total N input (g)	N in shrimp biomass (g)	Total organic N (g)	Total inorganic N (g)	Unaccounted N (g)	Initial N (g)	Balance (g)
25**	0.492	0.192[a]	0.178	0.099	0.183	0.160	0.492
30**	0.491	0.227[b]	0.161	0.071	0.193	0.160	0.491
35*	0.323	0.133	0.123	0.113	0.114	0.160	0.323
40*	0.323	0.135	0.115	0.129	0.105	0.160	0.323
	(%)	(%)	(%)	(%)	(%)	(%)	(%)
25**	100.0	39.0	36.2	20.2	37.1	32.5	100.0
30**	100.0	46.3	32.8	14.4	39.3	32.7	100.0
35*	100.0	41.0	38.0	35.0	35.4	49.4	100.0
40*	100.0	41.6	35.6	39.9	32.5	49.7	100.0

*Values are the means of five replicates; means within columns with the same letter and for the same week are not significantly different ($P<0.05$).

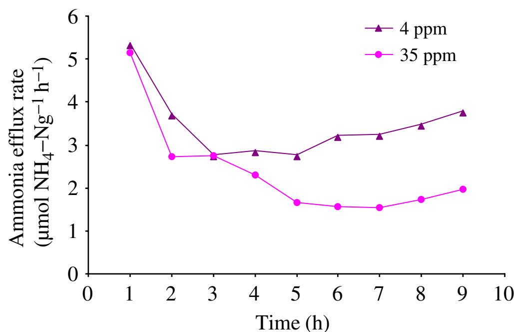

Figure 6 Initial (control) ammonia efflux rates (µmol NH_4-N$g^{-1}h^{-1}$) of juvenile *Litopenaeus vannamei* before and after acclimation to low salinity (36 g L^{-1} and 4.0 g L^{-1} respectively) at 28.5 °C. Values represent means of five replicate observations.

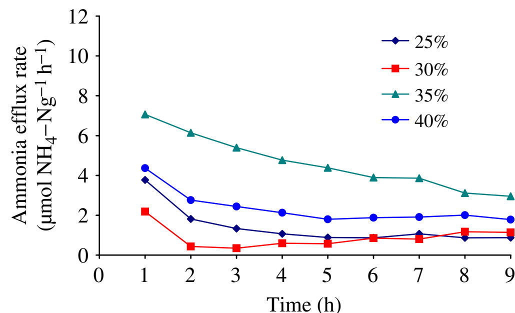

Figure 7 Ammonia efflux rate (µmol NH_4-N$g^{-1}h^{-1}$) of juvenile *Litopenaeus vannamei* maintained during 3 weeks in a zero-water exchange culture system at 30.1 °C and 4.0 g L^{-1}. Values represent means of five replicate observations.

regime to adjust the nutrient density of the diet and provide the same amount of protein did not offer any advantage or had an adverse effect on shrimp growth for the treatments compared, 25% vs. 30% protein and 35% vs. 40% protein, under these culture conditions. Kureshy and Davis (2002) demonstrated that a wide range of dietary protein levels can be used to produce maximum weight gain of *L. vannamei*; however, because of a restriction of feed intake, and thus protein intake, low-protein diets did not support maximum weight gain. On the other hand, in zero-water exchange culture systems, the use of low-protein diets is often recommended to facilitate the addition of carbon as a nutrient source for bacteria and to avoid accumulation of nitrogenous wastes within the system. Hence, even if a relatively higher amount of the low-protein diets was provided to shrimp under the isonitrogenous-feeding regime, the effect on water quality was less severe than that of the high-protein diets.

On the other hand, low survival observed for shrimp receiving the diets with 35% and 40% protein forced the termination of these treatments after 3 weeks in order to have enough animals for nitrogen analyses. Poor survival can be attributed to the influence of dietary protein content on nitrogen loading of the culture system, in spite of the isonitrogenous-feeding regime. Ammonia levels did not appear to be extremely high during this trial, always below the critical level of 3.95 mg L^{-1} reported for this species (Lin & Chen 2001). But nitrite, which is also considered to be a toxic metabolite for shrimp, accumulated

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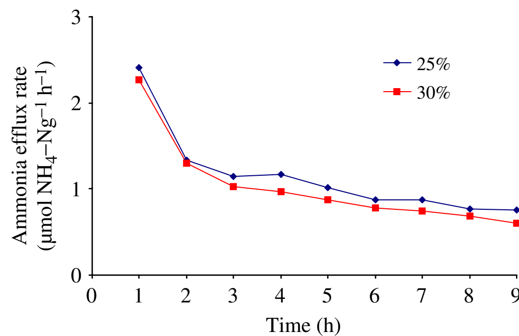

Figure 8 Ammonia efflux rate (μmol NH_4-Ng^{-1}h^{-1}) of juvenile *Litopenaeus vannamei* maintained during 4 weeks in a zero-water exchange culture system at 30.1 °C and 4.0 g L^{-1}. Values represent means of five replicate observations.

within the system and, for the 35% and 40% protein treatments, it reached a concentration of 5.0–6.0 mg L^{-1} after the second week, coinciding with high mortalities of the shrimp. By the end of the third week, the nitrite concentration was 7.36 mg L^{-1} in the 40% protein treatment and exacerbated mortalities forced the termination of both treatments. According to Lin and Chen (2003), salinity has an effect on nitrite toxicity; the 'safe level' for nitrite-N when rearing *L. vannamei* is 6.1, 15.2 and 25.7 mg L^{-1} in 15, 25 and 35 g L^{-1} of salinity respectively. In this study, the nitrite concentrations during the third week of the trial were near or even above the safe level cited for water at 15 g L^{-1}, but with water at 4.0 g L^{-1} this level is probably lower. Thus, high mortalities for these dietary treatments could be attributed to high concentrations of nitrite.

Highly unpredictable survival appears to be a common problem in low-salinity culture systems, but a combination of low salinity and zero or minimal water exchange in shrimp culture poses difficulties that require special attention, particularly water quality. In a similar low-salinity, zero-water exchange culture system, González-Félix *et al.* (2007) reported peak levels of ammonia of 5.8 mg L^{-1} for *L. vannamei* fed a 40% protein feed and of 4.0 mg L^{-1} for shrimp fed a 35% protein feed, which resulted in survival rates of 14.9% and 24.6% respectively. However, Samocha (2003) and Handy, Samocha, Patnaik, Gandy, Robinson and McKee (2004) reported ammonia concentrations of, respectively, 22.6 and 8.8 mg L^{-1} in seawater nursery cultures without any effect on shrimp growth or survival. Thus, the osmoregulatory stress of shrimp caused by a low-salinity environment, together with the high concentrations of nitrogenous metabolites, are probably responsible for the mortalities.

The nitrogen budget evaluation in the present study showed that 39.0–46.3% of the nitrogen input was incorporated into shrimp biomass (Table 3). These values are favourably higher compared with other reports for intensive shrimp culture of nearly 22% (Jackson, Preston, Thompson & Burford 2003) and 23–31% (Thakur & Lin 2003). However, our estimates are comparable to those reported by González-Félix *et al.* (2007) for similar experimental culture system and conditions, with 31.5–42.9% of the nitrogen incorporated into shrimp biomass. For semi-intensive culture systems, Páez-Osuna (2001) reported that up to 46.7% of the nitrogen could be recovered in shrimp biomass, and Islam, Sarker, Yamamoto, Wahab and Tanaka (2004) reported that 40% was recovered in shrimp biomass; thus, our observations are comparable to the values reported for shrimp culture under semi-intensive conditions. In our study, the isonitrogenous feeding was probably responsible for the higher estimated total organic nitrogen in the system, which ranged from 32.8% to 38.0%, being significantly higher for the 25% and 30% protein treatments, as could be expected. Nevertheless, in spite of the isonitrogenous feeding, higher total inorganic nitrogen, which ranged from 14.4% to 39.9%, was observed for the 40% and 35% protein treatments, and these values were also higher than the values reported by González-Félix *et al.* (2007). Thakur and Lin (2003) reported 14–53% of nitrogen trapped in sediments, and Jackson *et al.* (2003) up to 57% of nitrogen discharged in effluents. In both cases, a significant proportion could be attributed to organic nitrogen. For inorganic nitrogen, Thakur and Lin (2003) estimated that 14–28% of the input of nitrogen remained in this form in a closed system, and from 5.2% to 36.0% was unaccounted nitrogen. González-Félix *et al.* (2007) estimated 21.5–39.5% of unaccounted nitrogen, and in our study, it ranged from 32.5% to 39.3%, slightly higher for the 25% and 30% protein treatments. Nevertheless, Daniels and Boyd (1989) reported 55% nitrogen loss in brackish water fish ponds with very low exchange rates. Many studies attribute nitrogen losses to the removal of N_2 gas through denitrification and ammonia volatilization, which probably took place in our system; however, it is also likely that bacteria assimilated some nitrogen, although an estimation of the bacterial assimilation was not achieved.

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An increased ammonia efflux rate after handling shrimp was observed in all evaluations performed in this trial, but the rate decreased after a few hours of immersion; a similar response has been reported previously in other studies with shrimp (Regnault 1984; Gómez-Jiménez, González-Félix, Pérez-Velázquez, Trujillo-Villalba, Ezquerra-Brauer & Barraza-Guardado 2005; González-Félix *et al.* 2007). The mean ammonia efflux rate was higher for acclimated animals compared with shrimp before acclimation (3.19 μmol NH_4-Ng^{-1}h^{-1} at 4 g L^{-1} and 2.03 μmol NH_4-Ng^{-1}h^{-1} at 36 g L^{-1}). Gómez-Jiménez, Urias-Reyes, Vazquez-Ortiz and Hernandez-Watanabe (2004) reported that an abrupt reduction in salinity may considerably increase the ammonia efflux rate, even up to 30 μmol NH_4-Ng^{-1}h^{-1}, for animals transferred from seawater to 1.5 g L^{-1} water for 30 min. Then, after 3 weeks in a hypo-osmotic environment, *L. vannamei*, particularly those from the 35% and 40% dietary protein treatments, showed high ammonia efflux rates, similar to what was reported previously for this species (González-Félix *et al.* 2007). In spite of using an isonitrogenous-feeding regime, this study also evidenced an accumulation of nitrite and nitrate for the 35% and 40% protein treatments, as González-Félix *et al.* (2007) reported, although the accumulation of ammonia was less evident in this study. Furthermore, the efflux rates recorded during the third week of this study were also higher than the values reported by Gómez-Jiménez *et al.* (2005). This too could be explained by an increased ammonia efflux rate when shrimp were exposed to ambient nitrite > 5.02 mg NO_2-N L^{-1} in seawater of 30 g L^{-1}, as reported for *Penaeus monodon* (Fabricius) by Chen and Cheng (1995), or 5.77, 7.88 and 6.32 mg NO_2-N L^{-1} for the 30%, 35% and 40% protein treatments, respectively, at 4.6 g L^{-1}, as reported by González-Félix *et al.* (2007). In contrast, during week 4 of this trial the ammonia efflux rate of shrimp from the 25% and 30% protein treatments decreased compared with the control rate and the rate from the previous week, perhaps indicating the poor physiological state of animals that had been exposed to a hypo-osmotic environment and high nitrite concentrations for a prolonged period of time.

The results of this experiment showed that regardless of the protein content of the diet, animals following an isonitrogenous-feeding regime were able to use their ration efficiently for growth, as demonstrated from the analysis of IGR. However, in this study, nitrite concentration increased as dietary protein increased, despite the isonitrogenous loading of the system. As would be expected, when the nitrogen loading of the system increased, so did nitrogen excretion, which resulted in a deteriorated physiological state of the animals. Once again, this study confirms that closed systems are particularly susceptible to nitrogen loading; therefore, close attention should be paid to the level of protein being delivered and to the total amount of nitrogen being added to the system. Low-protein feeds have a tendency to perform better in zero-water exchange culture systems as they provide more carbon for heterotrophic bacteria and less nitrogen to be degraded and transformed into nitrogenous wastes.

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