

Nitrogen budget for a low salinity, zero-water exchange culture system: I. Effect of dietary protein level on the performance of *Litopenaeus vannamei* (Boone)

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Abstract

A 4-week study was conducted to evaluate the effects of different dietary protein levels (25%, 30%, 35% and 40%) on the growth and survival of juvenile *Litopenaeus vannamei* raised in a low salinity (4.6 g L⁻¹), zero-water exchange culture system, as well as on the nitrogen budget and ammonia efflux rate. No significant differences were observed among the dietary treatments for final weight, weight gain or survival of shrimp, although the best performance was observed in the 25% protein treatment group. Both weight and survival decreased as the dietary protein increased. Significant differences ($P < 0.05$) were observed in the ammonia concentration among dietary treatments during the first 2 weeks of the experiment. The highest concentration was measured in the 40% dietary protein treatment (5.88 mg NH₄-N L⁻¹). The nitrogen budget showed that the nitrogen loss increased as the dietary protein increased under the experimental conditions; the largest amount of nitrogen recovered as shrimp biomass (42.9%) was in the 25% protein treatment group, and the largest amount of unaccounted nitrogen (39.5%) was in the 40% protein treatment. Under these conditions, utilization of low-protein diets resulted in better performance, presumably because they provided more carbon for heterotrophic bacteria and reduced the nitrogen loading of the system.

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

Introduction

The potential negative impacts of commercial shrimp farms in coastal areas have generated a debate for some years. As an alternative to traditional coastal aquaculture, inland aquaculture using low-salinity well waters has presented itself as an advantageous and viable solution for the culture of several species. The Pacific white shrimp, *Litopenaeus vannamei* (Boone), can be grown in salinities as low as 0.5 g L⁻¹ (Smith & Lawrence 1990) and has a high market value, making it ideally suited for inland culture.

If inland aquaculture is to develop, it must adopt sustainable culture practices and minimize the problems found in traditional systems. Among the biggest threats to shrimp production are viral epidemics that result in high mortality. To minimize the introduction of pathogens into culture systems, farmers are implementing biosecurity measures such as minimizing the water exchange (Schuur 2003). Low water exchange systems will not only reduce the costs of water usage and pumping, but may also reduce feed costs, as natural productivity is higher. Waste products of the cultured animals as well as uneaten nutrients are retained in the system and drive primary production. These nutrients are incorporated by bacteria, phytoplankton and zooplankton, and, in turn, they serve as additional food sources for shrimp (Tacon, Cody, Conquest, Divakaran, Forster & Decamp 2002). Nitrifying and denitrifying bacteria, as well as phytoplankton, play

important roles in minimizing nitrogen loss because they reutilize nitrogen from uneaten feed, faeces and nitrogenous wastes (Burford, Preston, Glibert & Denison 2002).

Nitrogen inputs into shrimp ponds are mainly supplied in the form of protein in balanced feeds, but many systems are inefficient in transforming this nitrogen into shrimp biomass. Briggs and Funge-Smith (1994) estimated for intensive systems that 95% of the nitrogen input was in the form of feed and fertilizers while harvested shrimp accounted for only 24% of this nitrogen. As protein is a major limiting nutrient for shrimp growth, one of the most expensive components of the diet, and a potential pollutant, it is critical that protein retention is maximized by the shrimp (Kureshy & Davis 2002).

Protein retention by animals is dependent on a number of factors including food intake, level of protein in the feed, protein to energy ratio, amino acid balance as well as environmental factors. Research on protein requirements for shrimp, especially for *L. vannamei*, has focused on finding optimum dietary levels. Under laboratory conditions, reported levels have ranged from as low as 15% to as high as 46% of the diet (Aranyakananda & Lawrence 1994; Kureshy & Davis 2002). Hence, it is clear that a range of dietary protein can be used; however, there are limited data on the efficiency of retaining protein in the shrimp as well as the effect of various levels of dietary protein on water-quality parameters. Dietary protein that is not retained by the shrimp is either excreted as solid wastes (faecal matter and uneaten feed) or as soluble nitrogen, primarily in the form of ammonia. Both forms then add to the total loading of the culture system and can have detrimental effects on the culture environment.

The efficiency of a culture system in transforming nitrogen into shrimp biomass can be evaluated through a nitrogen budget. Nitrogen that is not incorporated into shrimp biomass can be found within the system in different forms, e.g., as dissolved organic or inorganic nitrogen. It can be trapped within suspended solids or sediments or it can be incorporated into bacteria, phytoplankton or zooplankton, and some may even be lost through ammonia volatilization (Thoman, Ingall, Davis & Arnold 2001). Most nitrogen is lost during discharge of effluents in traditional production systems; however, in closed systems nitrogen loss is considerably reduced (Thakur & Lin 2003).

If low-salinity zero-water exchange systems are to be supported and improved, a good understanding of

nitrogen dynamics and their interaction with the feed is necessary. This is important to assess not only from the nutritional viewpoint but also from a waste management perspective. The objective of this study was to evaluate the effect of different dietary protein levels on the growth and survival of juvenile *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 m²) with four dietary treatments (25%, 30%, 35% and 40% protein), each assigned to five aquaria in a completely random design. An additional aquarium (bottom area 0.49 m²) per dietary treatment was maintained under the same experimental conditions and with the same stocking density to keep shrimp for evaluation of ammonia excretion rates. Measurements of temperature and salinity were taken daily; the average values (\pm SD) were 29.0 \pm 0.2 °C and 4.6 \pm 0.2 g L⁻¹. Dissolved oxygen was also monitored daily and was maintained above 6.0 mg L⁻¹; pH was monitored twice a week and averaged 8.26 \pm 0.07. No water exchange was performed. Dechlorinated freshwater was added once a week to replace water losses due to evaporation.

Experimental diets

The four practical diets (Table 1) were formulated to contain 25%, 30%, 35% and 40% protein, and were manufactured in the laboratory of Auburn University, Department of Fisheries and Allied Aquaculture. They were prepared by mixing the dry ingredients in a mixer (Hobart, Troy, OH, USA) for 30 min. Subsequently, lipids were blended into the dry mix and hot water was then added to the mixture until the appropriate consistency for pelleting was obtained. After this, the diets were passed through a meat grinder and 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 commencement of experimental trials, when they were mechanically crumbled and sieved to the desired size. The daily ration fed to experimental shrimp was slowly reduced from approximately 12% of body weight to 5% by the

Table 1 Ingredient composition of experimental diets (g kg⁻¹ dry weight) fed to juvenile *Litopenaeus vannamei* during a 4-week trial

Ingredients	25%	30%	35%	40%
Menhaden fish meal*	156.25	187.50	218.75	250.00
Soybean meal†	248.00	306.00	365.00	424.00
Menhaden fish oil‡	21.50	27.40	33.30	39.20
Wheat starch§	316.55	228.40	139.25	49.10
Whole wheat§	200.00	200.00	200.00	200.00
Trace mineral premix¶	5.00	5.00	5.00	5.00
Vitamin premix	20.00	20.00	20.00	20.00
Vitamin C**	0.70	0.70	0.70	0.70
Calcium phosphate monobasic§	25.00	18.00	11.00	5.00
Soy lecithin††	5.00	5.00	5.00	5.00
Cholesterol§	2.00	2.00	2.00	2.00
Total	1000.00	1000.00	1000.00	1000.00

*Omega Protein, Hammond, LA, USA.

†De-hulled solvent extracted soybean meal, Southern Sates Cooperative, Richmond, VA, USA.

‡Omega Protein, Reedville, VA, USA.

§United States Biochemical Corporation, Cleveland, OH, USA.

¶g 100 g⁻¹ premix: cobalt chloride 0.004, cupric sulphate pentahydrate 0.250, ferrous sulphate 4.0, magnesium sulphate heptahydrate 28.398, manganous sulphate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193 and filler 53.428.

|| g kg⁻¹ premix: thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0, biotin 0.05, folic acid 0.18, vitamin B12 0.002, inositol 5.0, menadione 2.0, vitamin A acetate (20 000 IU g⁻¹) 5.0, vitamin D3 (400 000 IU g⁻¹) 0.002, dl- α -tocopheryl acetate (250 IU g⁻¹) 8.0, α -cellulose 865.266.

**250 mg kg⁻¹ active C supplied by Stay C[®], (L-ascorbyl-2-polyphosphate 25% Active C), Roche Vitamins, Parsippany, NJ, USA.

††Aqualipid 95, Central Soya Chemurgy Division, Fort Wayne, IN, USA.

end of the feeding trial, and it was fed to shrimp 15 times a day using automatic feeders. Feed inputs were adjusted daily for shrimp mortality and recorded in a logbook; at the end of the trial, the total amount of feed used per aquaria was known. Duplicate 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. (Mazatlan, Sinaloa, México). After acclimation 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, México) at 29 ± 1 °C and 34.5 ± 1.5 g L⁻¹, they were acclimated to 4 g L⁻¹.

This was done by gradually reducing salinity with previously aerated and dechlorinated freshwater in a seawater recirculating system of three 400 L tanks, at a constant rate of 1 g L⁻¹ h⁻¹ for 8 h day⁻¹ until the salinity of 4 g L⁻¹ was reached. Shrimp were fed a commercial postlarval feed (Camaronina, Agribrands Purina^{MR}, México) with 40% dietary protein for approximately 3 weeks until they attained an individual average size of 0.68 ± 0.01 g. Shrimp of similar size were blotted dry, weighed in groups of 10 and stocked into the aquaria. At the end of the feeding trial, shrimp were also 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

An initial shrimp sample (15 shrimp) and shrimp samples from each dietary treatment collected at the end of the trial were analysed for nitrogen content. Triplicate samples per treatment consisted of tissue from whole individual shrimp. They were thawed, dried in an oven at 75 °C during 24 h and macerated to obtain a fine dust, from which a single-pooled sample was prepared, and a 0.1 g duplicate sub-sample was analysed (MET990.03 AOAC 1984) in a Leco FP-528 nitrogen determinator (St Joseph, MI, USA). Duplicate samples of the experimental diets were analysed with the same equipment.

Inorganic nitrogen was determined as total ammonia nitrogen (NH₄⁺-N: Solarzano 1969; Spotte 1979a, b), nitrite (NO₂-N: Strickland & Parsons 1972; Spotte 1979a, b) and nitrate (NO₃-N: Mullen & Riley 1955; Spotte 1979a, b). Dissolved organic nitrogen (DON), suspended solids nitrogen (SSN) and settleable solids nitrogen (SetSN) were analysed using the method described by Solarzano and Sharp (1980) in which organic nitrogen is converted to nitrate during an oxidation with potassium persulphate under pressure. For the purposes of the analysis, the soluble fraction was defined as the organic material that passed through a 4.7 cm Whatman GF/C filter with a 1.2 µm particle retention. The suspended fraction was the organic material retained in the filter. The settleable fraction was evaluated only at the end of the experiment with a sample collected after manual agitation of the water in the aquaria. The difference between the suspended solids found in the sample before agitation and after agitation was defined as

the settable fraction. All water samples were collected in 500 mL acid-cleaned (10% HCl solution) plastic bottles, and kept in the dark at $-30\text{ }^{\circ}\text{C}$ until analysis. For the nitrogen budget, the ammonia, nitrite and nitrate concentrations were adjusted to express the total concentration per sample (500 mL) or aquaria (46.5 L), and then the nitrogen present was estimated for that volume considering their molecular weights.

Phytoplankton biomass was estimated at the beginning and at the end of the experiment using the method of Strickland and Parsons (1972) to monitor the concentration of chlorophyll *a*. Surface growth was measured by submerging two clean, dry and pre-weighed microscope slides suspended by a nylon monofilament along the sides of each aquarium. One of them was removed at week 3, while the second remained in the aquarium for the entire 4-week experiment. Upon removal, the substrates were dried overnight at $65\text{ }^{\circ}\text{C}$, weighed to determine the amount of surface growth and saved for subsequent nitrogen analyses.

The total nitrogen input (TNI) per aquaria was determined as

$$\begin{aligned} \text{TNI per aquaria} &= \text{total amount of feed given per aquaria} \\ &\times \text{average drymatter content of the feed} \\ &\times \text{fraction of nitrogen contained in dry feed} \end{aligned}$$

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

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

N_{SHRIMP} is the total amount of nitrogen incorporated into shrimp biomass at the end of the trial per aquaria. N_{TON} is the total amount of organic nitrogen present per aquaria, including DON, SSN and SetSN. N_{TIN} included the total amount of inorganic nitrogen present per aquaria, 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 present 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 aquaria (TNUA) was determined as

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

Ammonia efflux rate

Three ammonia efflux rates (control, and after 3 and 4 weeks) were determined during the experimental period following the protocols reported by Hagerman and Szaniawska (1994), Schmitt and Uglow (1997), Gómez-Jiménez, Urias-Reyes, Vazquez-Ortiz and Hernandez-Watanabe (2004). Before initiation of the experiment, baseline ammonia efflux rates (control) were determined from a random sample of eight juvenile shrimp ($0.67 \pm 0.26\text{ g}$) kept unfed for 24 h. Each one of the shrimp was stocked into individual round glass aquaria measuring 0.2 m (diameter) \times 0.16 m (height) filled with 250 mL of clean 4.6 g L^{-1} water at $28\text{ }^{\circ}\text{C}$ (control and 3 weeks), or 300 mL of similar water during the final evaluation (4 weeks). Aquaria were acid cleaned (10% HCl solution) and rinsed with distilled water. Gentle aeration was provided during the 9-h evaluation period to ensure good water mixing and adequate oxygen levels within the aquaria. Water samples for the measurement of ammonia efflux rates were collected hourly after 1 h and during the 9 h of immersion. Three aquaria without animals were used as a blank. Weight-specific ammonia efflux rates were calculated considering individual shrimp weight, the time between sample collections and the water volume. In this case, total ammonia was quantified using a flow injection/gas diffusion (FIA) technique, adapted from Clinch, Worsfold and Sweeting (1988), and Hunter and Uglow (1993). Total ammonia analysed with FIA refers to the sum of NH_3 and NH_4^+ and is referred to as ammonia. On weeks 3 and 4 of the experiment, eight shrimp (mean weight 2.06 ± 0.58 and $2.86 \pm 0.73\text{ g}$ respectively) per dietary treatment were sampled from an additional aquarium maintained under the same experimental conditions with animals destined for the evaluation of ammonia efflux rates. These shrimp were discarded after all measurements were performed.

Statistical analysis

Shrimp performance was evaluated through final weight, weight gain (expressed as a per cent of initial weight) and survival. Survival was transformed by arcsine square root before statistical analysis. All data were analysed using a one-way analysis of var-

iance (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 (V8 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, or for final weight, weight gain and survival at the end of the experiment. However, a trend towards better biological performance of shrimp was observed as the dietary protein content decreased under these experimental conditions; thus, shrimp fed a 25% protein diet showed the greatest final weight, weight gain and survival and those fed a 40% protein diet showed the lowest values for the same parameters (Table 2).

Nitrogen metabolites and budget

Total ammonia nitrogen showed significant differences among treatments during the first 2 weeks of the trial; the ammonia concentration in the water column was directly influenced by the dietary protein level (Fig. 1). The highest ammonia concentration was observed during the second week for the treatment offered 40% of dietary protein ($5.88 \text{ mg NH}_4\text{-NL}^{-1}$). During the third and the fourth weeks of the trial, the ammonia concentration decreased at the same time that nitrite and nitrate concentration started to increase, but no significant differences among treatments were observed for these nitrogen metabolites. The nitrite concentration was higher for dietary treatments with 35% and 40% protein dur-

ing the third week of the trial (7.88 and $6.32 \text{ mg NO}_2\text{-NL}^{-1}$ respectively; Fig. 2). The nitrate concentration was higher for the same treatments also during the third week of this trial (8.39 and $8.67 \text{ mg NO}_3\text{-NL}^{-1}$ respectively; Fig. 3).

Dissolved organic nitrogen showed no significant differences among treatments; the highest values were registered during the third week of the trial for the 35% and 40% dietary protein treatments (9.15 and $8.00 \text{ mg NO}_3\text{-NL}^{-1}$ respectively; Fig. 4). Suspended solids nitrogen followed a similar trend as DON; treatments with 35% and 40% dietary protein showed the highest concentrations (3.00 and $2.99 \text{ mg NO}_3\text{-NL}^{-1}$ respectively) during the third week of the trial (Fig. 5). Setttable solids nitrogen observed at the end of the trial showed no differences among treatments, with concentrations of 3.19 ± 0.60 , 4.08 ± 0.27 , 3.09 ± 0.67 and $3.02 \pm 0.86 \text{ mg NO}_3\text{-NL}^{-1}$ for the 25%, 30%, 35% and 40% dietary protein treatments respectively.

Nitrogen and protein contents for initial shrimp (71.89% of protein, on a dry weight basis) and final shrimp were determined at the end of the trial. A total of 73.03% , 72.55% , 72.70% and 68.94% of protein contents were recorded for animals fed 25%, 30%, 35% and 40% of dietary protein. Additionally, all experimental diets were analysed and a protein content of 27.63% , 32.35% , 37.02% and 42.06% was recorded for the 25%, 30%, 35% and 40% protein diets respectively.

Chlorophyll *a* levels were close to zero during the second and the fourth week of this trial, indicating an extremely low phytoplankton level; thus, nitrogen uptake by phytoplankton was considered to be negligible. Although an electronic scale accurate to four decimal places (Mettler AE240, Hightstown, NJ, USA) was used to record surface growth accumulation on the microscope slides, it was not possible to quantify bacterial growth. Consequently, an estimate of bacterial assimilation of inorganic nitrogen or bacterial degradation of organic nitrogen was not

Table 2 Initial and final weight, weight gain and survival of juvenile *Litopenaeus vannamei* fed different dietary protein levels in a low-salinity zero-water exchange culture system*

Treatment (% dietary protein)	Initial weight (g)	Final weight (g)	Weight gain (% initial weight)	Survival (%)
25	0.68 ± 0.01	3.11 ± 0.29	457.14 ± 40.02	66.00 ± 42.42
30	0.67 ± 0.00	2.89 ± 0.22	431.15 ± 31.03	25.80 ± 24.38
35	0.68 ± 0.01	2.80 ± 0.28	416.69 ± 39.87	24.64 ± 26.44
40	0.68 ± 0.01	2.31 ± 0.46	343.23 ± 73.41	14.93 ± 15.74

*Values are means of five replicates \pm SD.

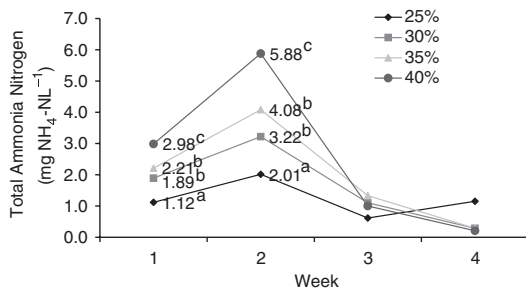


Figure 1 Total ammonia nitrogen (mg NH₄-NL⁻¹) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent the means of five replicate observations. Means within weeks with the same letter are not significantly different ($P < 0.05$).

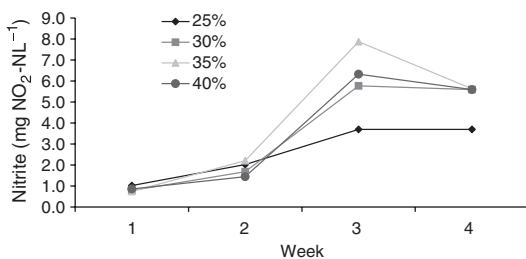


Figure 2 Total nitrite (mg NO₂-NL⁻¹) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent the means of five replicate observations.

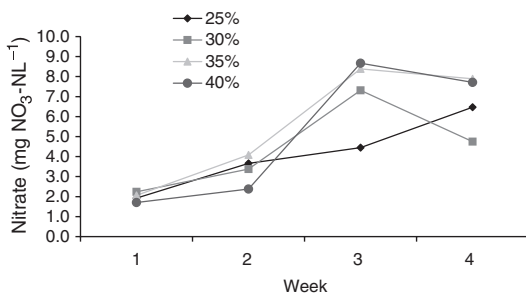


Figure 3 Total nitrate (mg NO₃-NL⁻¹) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent the means of five replicate observations.

achieved, although these processes were probably taking place within the aquaria.

The nitrogen balance (Table 3) showed that the most significant incorporation of nitrogen into shrimp biomass, which ranged from 31.5% to 42.9% (0.209–

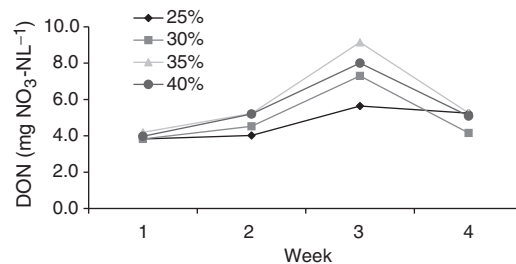


Figure 4 Dissolved organic nitrogen (DON: mg NO₃-NL⁻¹) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent the means of five replicate observations.

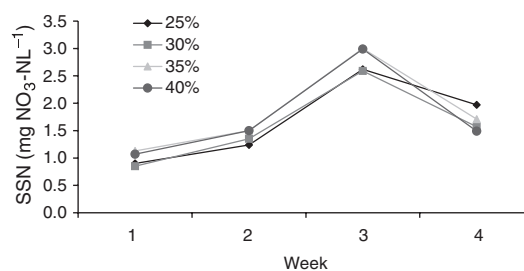


Figure 5 Suspended solids nitrogen (SSN: mg NO₃-NL⁻¹) during a 4-week growth trial evaluating four dietary protein levels fed to *Litopenaeus vannamei* raised in a low-salinity zero-water exchange culture system. Values represent the means of five replicate observations.

0.278 g), took place in the treatment with 25% dietary protein and decreased as dietary protein increased, in a manner similar to N_{TIN}, which ranged from 14.7% to 24.4% (0.097–0.158 g), although no significant differences were observed for these components. In contrast, N_{TON} ranged from 26.3% to 32.8% (0.170–0.247 g), but showed no particular trend with respect to the amount of dietary protein provided. In fact, the dietary treatment with 30% protein showed a significantly higher N_{TON} than the rest of the treatments. Total nitrogen unaccounted per aquaria ranged from 21.5% to 39.5% (0.139–0.265 g), also increasing as dietary protein increased, but no significant differences among treatments were observed. As for N_{INITIAL}, no differences among treatments were observed either; this component ranged from 12.3% to 15.2% (0.097–0.098 g).

Ammonia efflux rates

The control ammonia efflux rate showed the highest value after the first hour following transfer of the an-

Table 3 Nitrogen budget for a low-salinity zero-water exchange culture system where *Litopenaeus vannamei* was raised with four dietary protein levels (25%, 30%, 35% and 40%) during a 4-week growth trial*

Treatment (% dietary protein)	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	0.648	0.278	0.170 ^a	0.158	0.139	0.098	0.648
30	0.755	0.268	0.247 ^b	0.140	0.197	0.097	0.755
35	0.795	0.294	0.179 ^a	0.155	0.265	0.098	0.795
40	0.663	0.209	0.192 ^a	0.097	0.262	0.097	0.663
(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
25	100.0	42.9	26.3	24.4	21.5	15.2	100.0
30	100.0	35.5	32.8	18.6	26.1	12.9	100.0
35	100.0	37.0	22.5	19.5	33.3	12.3	100.0
40	100.0	31.5	29.0	14.7	39.5	14.6	100.0

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

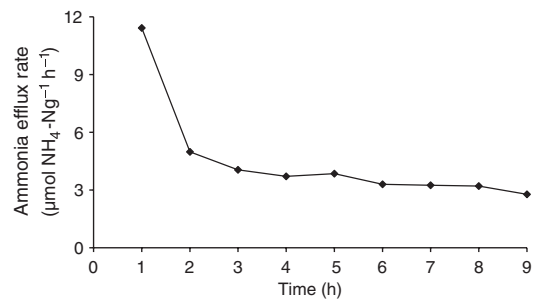


Figure 6 Control ammonia efflux rate ($\mu\text{mol NH}_4\text{-N g}^{-1} \text{h}^{-1}$) of juvenile *Litopenaeus vannamei* maintained in a zero-water exchange culture system at 29 °C and 4.6 g L⁻¹. Values represent the means of eight replicate observations.

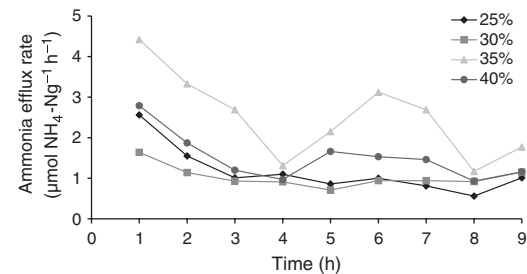


Figure 7 Ammonia efflux rate ($\mu\text{mol NH}_4\text{-N g}^{-1} \text{h}^{-1}$) of juvenile *Litopenaeus vannamei* maintained for 3 weeks in a zero-water exchange culture system at 29 °C and 4.6 g L⁻¹. Values represent the means of eight replicate observations.

imals to individual experimental aquaria, almost certainly indicating a handling effect (Fig. 6). These values were not included, therefore, in the calculation of the mean ammonia excretion rate of $3.45 \pm 0.17 \mu\text{mol NH}_4\text{-N g}^{-1} \text{h}^{-1}$ of juvenile *L. vannamei* kept at 29 °C and 4.6 g L⁻¹. Figures 7 and 8

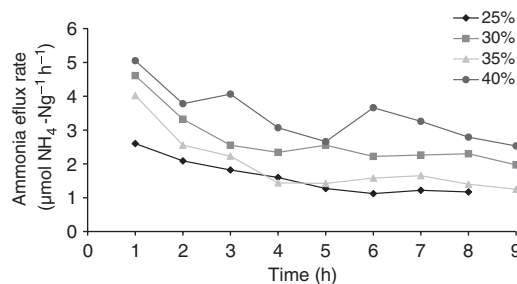


Figure 8 Ammonia efflux rate ($\mu\text{mol NH}_4\text{-N g}^{-1} \text{h}^{-1}$) of juvenile *Litopenaeus vannamei* maintained for 4 weeks in a zero-water exchange culture system at 29 °C and 4.6 g L⁻¹. Values represent means of eight replicate observations.

show ammonia efflux rates after 3 and 4 weeks of exposure to culture conditions. Similar to the control conditions, the highest excretion rates were measured after the first hour; once these values were excluded, all groups in general exhibited a decreasing ammonia efflux rate with time. The lowest efflux rates were recorded for shrimp fed a 30% and 25% protein diet during the third and fourth week of this trial.

Discussion

Shrimp culture in low-salinity water is considered to be a feasible alternative for euryhaline species such as *L. vannamei* (Davis, Saoud, McGraw & Rouse 2002; Decamp, Cody, Conquest, Delanoy & Tacon 2003; McIntosh & Fitzsimmons 2003), even under low or zero-water exchange conditions (McIntosh, Samocha, Jones, Lawrence, Horowitz & Horowitz 2001; Burford, Thompson, McIntosh, Bauman & Pearson 2003; Decamp *et al.* 2003; Thakur & Lin 2003). However, as

the animals are already under a stressful condition, maintenance of good water quality should be emphasized to promote adequate shrimp growth and survival (Decamp *et al.* 2003; Thakur & Lin 2003). The ability to maintain good water quality depends on a number of factors including nutrient loading and the assimilation capacity of the system. During this trial, water quality parameters such as temperature, dissolved oxygen and pH were maintained at good levels for this species. However, the salinity (4.6 g L^{-1}) was maintained at very low levels, demonstrating the capacity of this marine species to adapt to a low-salinity environment.

In spite of the absence of significant differences among treatments for the biological performance parameters evaluated, numerically better growth (3.11 g, 457.14% of the initial weight) and survival (66%) was observed among shrimp fed a 25% protein diet, as compared with those maintained on the 40% diet, which were numerically the smallest (2.31 g, 343.23% of the initial weight) and showed the lowest (14.93%) survival. Tacon *et al.* (2002) reported a survival ranging from 19% to 79.3% for *L. vannamei* raised in an outside zero-water exchange system with seawater for 8 weeks, as well as percentages of weight gain ranging from 554.7% to 1098.2%. In addition, they reported percentages of weight gain ranging from 130.2% to 323.8% for animals raised in an indoor flow-through culture system during the same period of time. They concluded that the superior performance of shrimp reared in the outside zero-water exchange system was primarily due to their ability to obtain additional nutrients from food organisms endogenously produced within this culture system. Shrimp growth of this experiment carried out in indoor zero-water exchange culture conditions was better than shrimp growth reported by Tacon *et al.* (2002) for an indoor flow-through culture system with no natural productivity, but worse than shrimp growth raised in an outside zero-water exchange system with primary production. The growth rates of shrimp in the present trial were intermediate to the values reported by Tacon *et al.* (2002), presumably because the primary production in our system was intermediate to a clear flow-through system and an outdoor tank system, i.e., the shrimp had access to bacterial floc but not phytoplankton.

Highly variable survival is quite often a problem in low-salinity culture systems. One reason for variable survival could be due to variations in water quality and the increased sensitivity of the shrimp to some water-quality parameters. In the present experiment,

the biological performance of shrimp, particularly survival, appeared to be influenced by the dietary protein content and consequently nitrogen loading of the culture system. As would be expected, nitrogenous wastes (ammonia, nitrite and nitrate) within the system increased as the dietary protein and nitrogen loading of the system increased (Figs. 1–3). Ammonia reached a concentration of $5.88 \text{ mg NH}_4\text{-NL}^{-1}$ for the 40% protein treatment by the end of the second week. This coincided with the high mortality rates observed from that day onwards, especially for the 40% and 35% dietary protein treatments. High mortality rates associated with high ammonia levels have been reported for *L. vannamei* (Racotta & Hernández-Herrera 2000) and *Marasupeneus japonicus* (Bate) (Lin, Thuét, Trilles, Mounet-Guillaume & Charmantier 1993). Nitrite is also considered to be a toxic metabolite for shrimp; it began to accumulate within the system after the second week. This is a typical cycling of nitrogen compounds that occurs as a result of the establishment of nitrifying bacteria that carry out the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and nitrate (NO_3^-), as reported previously (Decamp *et al.* 2003).

Lin and Chen (2003) evaluated the “safe level” for nitrite-N when rearing *L. vannamei* juveniles at different salinities and estimated it to be 6.1, 15.2 and 25.7 mg L^{-1} in 15, 25 and 35 g L^{-1} , respectively, demonstrating the effect of salinity on nitrite toxicity. In this study, the highest registered nitrite concentrations of 5.77, 7.88 and $6.32 \text{ mg NO}_2\text{-NL}^{-1}$ for the 30%, 35% and 40% dietary protein treatments, respectively, during the third week of the trial were near or above the safe level cited for 15 g L^{-1} , but at 4.6 g L^{-1} this level is probably lower. Thus, high mortalities for these dietary treatments could be attributed not only to high concentrations of ammonia but also high concentrations of nitrite.

Tacon *et al.* (2002) also observed a progressive deterioration in water quality during the last 2 weeks of culture in an outside zero-water exchange culture system with seawater evidenced by a decrease in pH and a marked increase in nitrite following a peak in ammonia. As with all biological systems, the ecosystem is required to process wastes in a closed zero-water exchange system, and it is only able to withstand a certain level of nutrient input and shrimp biomass before the system crashes and compromises shrimp growth and survival. During this trial, a pH decline was not observed, indicating that the buffering capacity of the system was not exceeded. The increase in nitrite after a peak of ammonia was evident

(Figs 1 and 2), and would be considered to be typical of nitrification systems that are adjusting to a new load. Differences in the timing of nutrient peaks in the present study and that of Tacon *et al.* (2002) are probably due to their system being driven by both phytoplankton and bacteria. In outdoor systems, bacterial and phytoplankton growth is encouraged by favourable conditions allowing nutrients and excretory and digestive metabolites to be depleted from the water column by a variety of these organisms (McNeil 2000). As nutrient re-cycling was limited to bacteria, accumulation of metabolites in the present study was more pronounced, particularly for the treatments with a higher protein content where nutrient loading was the highest. Thus, high concentrations of nitrogenous metabolites, together with the effect of low-salinity water adding further osmoregulatory stress for shrimp, were probably responsible for the abundant mortalities.

It is known that dietary protein is the major limiting nutrient for growth and one of the most expensive. Kurshy and Davis (2002) evaluated the protein requirement for the maximum growth of juvenile *L. vannamei* and estimated it to be 46.4 g dietary protein per kilogram body weight per day ($\text{g DP kg}^{-1} \text{BW day}^{-1}$) when fed a 32% protein diet and 43.4 $\text{g DP kg}^{-1} \text{BW day}^{-1}$ when fed a 48% protein diet. They noted that a wide range of dietary protein levels could be used to lead to maximum weight gain of juvenile and subadult shrimp, but due to a restriction of feed intake and consequently protein intake, the low-protein diet (16% protein) did not support maximum weight gain.

Quite often, people overlook the interaction of dietary nutrient levels and feed intake. Hence, a fixed ration is offered irrespective of the nutrient density of the diet. In the case of dietary protein, aquatic animals are well adapted to using protein as an energy source. Hence, when the daily intake exceeds the requirement or when the non-protein energy is limiting, protein is easily metabolized as an energy source and nitrogen is excreted. 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, which will occur if the animals are overfed. Nevertheless, *L. vannamei*, an omnivorous species, can grow adequately on diets with as low as 20.2% dietary protein (Velasco, Lawrence, Castille & Obaldo 2000) as long as adequate consumption rates are maintained.

In the present study, the largest concentration of DON and SSN was observed during the third week of

the trial, particularly for the treatments with 35% and 40% dietary protein, although no significant differences were evidenced for them or SetSN among treatments. The nitrogen budget showed that, for the 25% dietary protein treatment, 42.9% of the nitrogen input was incorporated into shrimp biomass, compared with only 31.5% for the 40% treatment. Jackson, Preston, Thompson and Burford (2003) evaluated the nitrogen budget for an intensive shrimp farm, and found that only 22% of the nitrogen was incorporated into shrimp biomass, while Thakur and Lin (2003) found that 23–31% of the nitrogen was incorporated by shrimp raised in concrete tanks for a period of 90 days without water exchange. Thoman *et al.* (2001) registered 25.4–31.5% of nitrogen recovered in fish biomass raised in a closed, recirculating mariculture system. Hence, the values for nitrogen incorporation in this study are comparable with those reported by other authors. The total organic nitrogen in the system ranged from 22.5% to 32.8%. Other studies have reported losses ranging from 14% to 53% of nitrogen trapped in sediments (Thakur & Lin 2003), which could be considered another form of organic nitrogen, as well as up to approximately 57% of nitrogen discharged in effluents (Jackson *et al.* 2003), from which a significant part could be organic nitrogen. The total inorganic nitrogen present in our system at the end of the trial ranged from 14.7% to 24.4%. Another study carried out by Thakur and Lin (2003) in a closed system provided very similar data ranging from 14% to 28%. They also reported that unaccounted nitrogen ranged from 5.2% to 36.0% of the total input in their system, while Thoman *et al.* (2001) reported a nitrogen loss of 9–21% from their recirculating system, and suggested that removal of N_2 gas through denitrification was the most likely explanation for that loss, because ammonia volatilization rates based on the thin-film gas exchange model indicate that this process probably contributes very little to the nitrogen deficit of culture systems. Previously, Daniels and Boyd (1989) had reported 55% nitrogen loss in brackish water fish ponds with very low exchange rates ($\approx 10\%$ over a 5-month period), which they attributed to denitrification and volatilization, but Jackson *et al.* (2003) mentioned that it is also possible that some unaccounted nitrogen is sequestered in the sediment through accumulation of organic sludge. For this experimental system, unaccounted nitrogen varied from 21.5% to 39.5%; nitrogen losses could be attributed not only to removal of N_2 gas through denitrification, and possibly a small amount of ammonia volatilization, but

also it is very likely that a certain quantity of nitrogen was assimilated by bacteria. Unfortunately, an estimation of bacterial assimilation could not be performed.

Concerning the ammonia efflux rates, several studies have reported increased rates after handling animals (Spaargaren, Richard & Ceccaldi 1982; Regnault 1984; Gómez-Jiménez, González-Félix, Pérez-Velázquez, Trujillo-Villalba, Ezquerro-Brauer & Barraza-Guardado 2005); such a response was also observed in all evaluations performed during this trial. The mean ammonia efflux rate for the control group, $3.45 \mu\text{mol NH}_4\text{-N g}^{-1} \text{h}^{-1}$, is higher than the $2.18 \mu\text{mol NH}_4\text{-N g}^{-1} \text{h}^{-1}$ reported by Gómez-Jiménez *et al.* (2005) in juvenile shrimp of the same species at 28°C and 37 g L^{-1} . The control rate recorded in this study reflects the osmoregulatory pattern reported by Diaz, Farfan, Sierra and Re (2001) for this species. In a hypo-osmotic environment, *L. vannamei* has shown a hyper-regulatory mechanism leading to an increased ammonia efflux rate, as recorded in this study. Similarly, efflux rates recorded during weeks 3 and 4 are higher than the values reported previously by Gómez-Jiménez *et al.* (2005) on days 14 and 21 of their experiment. Chen and Cheng (1995) reported an increased ammonia excretion rate in *Penaeus monodon* (Fabricius) when exposed to ambient nitrite $> 5.02 \text{ mg NO}_2\text{-N L}^{-1}$ in seawater of 30 g L^{-1} , whereas in this study at 4.6 g L^{-1} the highest registered nitrite concentrations during week 3 were 5.77, 7.88 and $6.32 \text{ mg NO}_2\text{-N L}^{-1}$ for the 30%, 35% and 40% dietary protein treatments respectively. In contrast, during weeks 3 and 4 of this trial, the ammonia efflux rate of shrimp in all dietary treatments decreased compared with the control rate, perhaps as a result of physiological adjustments carried out by animals exposed for a longer period of time to a hypo-osmotic environment. Evidently, exposure to more than one stressful environmental factor (low salinity and high nitrite concentrations) affects shrimp nitrogen metabolism and may explain differences in excretion rates, although further research is required to clarify this.

Based on the results of this experiment, there is a clear relationship between dietary protein and nitrogen excretion rates. As would be expected, when the dietary protein level increases, so does nitrogen excretion and consequently nitrogen loading. Hence, when dealing with closed systems one needs to consider both the desired level of protein to be delivered to the animal as well as the amount of nitrogen entering the culture system. One advantage of low-protein feeds is that they will provide more carbon for heterotrophic bacteria and less nitrogen that will need to be

degraded. Therefore, utilization of diets with a low-dietary protein content resulted in better performance, presumably due to reduced nitrogen loading of this zero-water exchange culture system.

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References

- Aranyakananda P. & Lawrence A.L. (1994) Efectos de la tasa de ingestión sobre los requerimientos alimenticios en proteína y energía y la relación óptima proteína-energía para *Penaeus vannamei*. In: *Proceedings of the II International Symposium on Aquatic Nutrition, Avances en Nutrición Acuicola II* (ed. by R.E. Mendoza, L.E. Cruz-Suárez & D. Ricque), pp. 157–169. Nuevo Leon, Monterrey, Mexico.
- Association of Official Analytical Chemists. (1984) *Official Methods of Analysis*. Association of Analytical Chemists, Arlington, VA, USA.
- Briggs M.R.P. & Funge-Smith S.J. (1994) A nutrient budget of some intensive marine shrimp ponds in Thailand. *Aquaculture and Fisheries Management* **25**, 789–811.
- Burford M.A., Preston N.P., Glibert P.M. & Dennison W.C. (2002) Tracing the fate of ^{15}N -enriched feed in an intensive shrimp system. *Aquaculture* **206**, 199–216.
- Burford M.A., Thompson P.J., McIntosh R.P., Bauman R.H. & Pearson D.C. (2003) Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. *Aquaculture* **219**, 393–411.
- Chen J.C. & Cheng S.Y. (1995) Hemolymph oxygen content, oxyhemocyanin, protein levels and ammonia excretion in the shrimp *Penaeus monodon* exposed to ambient nitrite. *Journal of Comparative Physiology B* **164**, 530–535.
- Clinch J.R., Worsfold P.J. & Sweeting E.W. (1988) An automated spectrophotometric field monitor for water quality parameters: determination of ammonia. *Analytica Chimica Acta* **214**, 401–407.
- Daniels H.V. & Boyd C.E. (1989) Chemical budgets for polyethylene-lined, brackish water ponds. *Journal of the World Aquaculture Society* **20**, 53–60.
- Davis D.A., Saoud I.P., McGraw W.J. & Rouse D.B. (2002) Considerations for *Litopenaeus vannamei* reared in low-salinity water. In: *Proceedings of the VI International Symposium on Aquatic Nutrition, Avances en Nutrición Acuicola VI* (ed. by L.E. Cruz-Suárez, D. Ricque-Marie, M. Tapia-Salazar, M.G. Gaxiola-Cortés & N. Simoes), pp. 74–90. Cancun, Quintana Roo, Mexico.

- Decamp O., Cody J., Conquest L., Delanoy G. & Tacon A.G. (2003) Effect of salinity on natural community and production of *Litopenaeus vannamei* (Boone 1931), within experimental zero-water exchange culture systems. *Aquaculture Research* **34**, 345–355.
- Diaz E., Farfan C., Sierra E. & Re A.D. (2001) Effects of temperature and salinity fluctuation on the ammonium excretion and osmoregulation of juveniles of *Penaeus vannamei*, Boone. *Marine and Freshwater Behaviour and Physiology* **34**, 93–104.
- Gómez-Jiménez S., Urias-Reyes A., Vazquez-Ortiz E. & Hernandez-Watanabe G. (2004) Ammonia efflux rates and free amino acid levels in *Litopenaeus vannamei* postlarvae during sudden salinity changes. *Aquaculture* **233**, 573–581.
- Gómez-Jiménez S., González-Félix M.L., Pérez-Velázquez M., Trujillo-Villalba D.A., Ezquerro-Brauer I.R. & Barraza-Guardado R. (2005) Effect of dietary protein level on growth, survival and ammonia efflux rate of *Litopenaeus vannamei* (Boone) raised in a zero-water exchange culture system. *Aquaculture Research* **36**, 834–840.
- Hagerman L. & Szaniawska A. (1994) Haemolymph nitrogen compounds and ammonia efflux rates under anoxia in the brackish water isopod *Saduria entomon*. *Marine Ecology Progress* **103**, 285–289.
- Hunter D.A. & Uglow R.F. (1993) A technique for the measurement of total ammonia in small volumes of seawater and haemolymph. *Ophelia* **37**, 31–40.
- Jackson C., Preston N., Thompson P.J. & Burford M. (2003) Nitrogen budget and effluent nitrogen components at an intensive shrimp farm. *Aquaculture* **218**, 397–411.
- Kureshy N. & Davis D.A. (2002) Protein requirement for maintenance and maximum weight gain for the Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture* **204**, 125–143.
- Lin Y.C. & Chen J.C. (2003) Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. *Aquaculture* **224**, 193–201.
- Lin H.P., Thuet P., Trilles J.P., Mounet-Guillaume R. & Charmantier G. (1993) Effects of ammonia on survival and osmoregulation of various development stages of the shrimp *Penaeus japonicus*. *Marine Biology* **117**, 591–598.
- McIntosh D. & Fitzsimmons K. (2003) Characterization of effluent from an inland, low-salinity shrimp: what contribution could this water make if used for irrigation. *Aquacultural Engineering* **27**, 147–156.
- McIntosh D., Samocha T.M., Jones E.R., Lawrence A.L., Horowitz S. & Horowitz A. (2001) Effects of two commercially available low-protein diets (21% and 31%) on water and sediment quality, and on the production of *Litopenaeus vannamei* in an outdoor tank system with limited water discharge. *Aquaculture Engineering* **25**, 69–82.
- McNeil R. (2000) Zero exchange, aerobic, heterotrophic systems: key considerations. *Global Aquaculture Advocate* **3**, 72–76.
- Mullen J.D. & Riley J.P. (1955) The spectrophotometric determination of nitrate in natural waters, with particular reference to sea-water. *Analytica Chimica Acta* **12**, 464–480.
- Racotta I.S. & Hernández-Herrera R. (2000) Metabolic responses of the white shrimp, *Penaeus vannamei*, to ambient ammonia. *Comparative Biochemistry and Physiology* **125**, 437–443.
- Regnault M. (1984) Salinity induced changes in ammonia excretion rate of the shrimp *Crangon crangon* over a winter tidal cycle. *Marine Ecology Progress Series* **20**, 119–125.
- SAS institute Inc. (1999–2000) *The SAS System for Windows, Software Release 8.1*. Cary, NC, USA.
- Schmitt A.S.C. & Uglow R.F. (1997) Effects of ambient ammonia levels on blood ammonia, ammonia excretion and heart and scaphognathite rates of *Nephrops norvegicus*. *Marine Biology* **127**, 411–418.
- Schuur A.M. (2003) Evaluation of biosecurity applications for intensive shrimp farming. *Aquacultural Engineering* **28**, 3–19.
- Smith L.L. & Lawrence A.L. (1990) Feasibility of penaeid shrimp culture in inland saline groundwater-fed ponds. *The Texas Journal of Science* **42**, 3–12.
- Solarzano L. (1969) Determination of ammonia in natural waters by the phenylhypochlorite method. *Limnology and Oceanography* **14**, 799–801.
- Solarzano L. & Sharp J. (1980) Determination of total dissolved organic nitrogen. *Limnology and Oceanography* **25**, 751–754.
- Spaargaren D.H., Richard P. & Ceccaldi H.J. (1982) Excretion of nitrogenous products by *Penaeus japonicus* Bate in relation to environmental osmotic conditions. *Comparative Biochemistry and Physiology* **72**, 673–678.
- Spotte S. (1979a) *Fish and Invertebrate Culture: Water Management in Closed Systems*. Wiley, New York, USA, 179pp.
- Spotte S. (1979b) *Seawater Aquariums: The Captive Environment*. Wiley, New York, USA, 413pp.
- Strickland J.D.H. & Parsons T.R. (1972) *A Practical Handbook of Seawater Analysis*. Bulletin 167, Fisheries Research Board of Canada, Ottawa, Canada 310pp.
- Tacon A.G.J., Cody J.J., Conquest L.D., Divakaran S., Forster L.P. & Decamp O.E. (2002) Effect of culture system on the nutrition and growth performance of Pacific white shrimp *Litopenaeus vannamei* (Boone) fed different diets. *Aquaculture Nutrition* **8**, 121–137.
- Thakur D.P. & Lin C.K. (2003) Water quality and nutrient budget in closed shrimp (*Penaeus monodon*) culture systems. *Aquacultural Engineering* **27**, 159–176.
- Thoman E.S., Ingall E.D., Davis D.A. & Arnold C.R. (2001) A nitrogen budget for a closed, recirculating mariculture system. *Aquacultural Engineering* **24**, 195–211.
- Velasco M., Lawrence A.L., Castille F.L. & Obaldo L.G. (2000) Dietary protein requirement for *Litopenaeus vannamei*. In: *Proceedings of the V International Symposium on Aquatic Nutrition, Avances en Nutrición Acuicola V* (ed. by L.E. Cruz-Suárez, D. Ricque-Marie, M. Tapia-Salazar, M. Olvera-Novoa & R. Civera-Cerecedo), pp. 181–192. Merida, Yucatán, México.