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Nitrogen and phosphorous budgets for integrated culture of whiteleg shrimp *Litopenaeus vannamei* with red seaweed *Gracilaria corticata* in zero water exchange system

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Abstract

In this study, a 2×3 factorial design with two levels of shrimp density (25 and 50 shrimp per m^2) and three levels of red algae density (0, 200 and 400g per m^2) was applied to calculate nitrogen and phosphorous budgets in the integrated culture of *Litopenaeus vannamei* with *Gracilaria corticata* during 45 days in a zero water exchange system. Juveniles of *L. vannamei* (5.82±0.11 g) and *G. corticata* were cultured in 18 round 1 m^3 poly ethylene tanks. Water temperature, dissolved oxygen (DO), pH and salinity were measured once every 3 days. Results indicated that shrimp density had a significant effect on pH and DO in the morning and in the afternoon. The algal density didn't have a significant effect on pH and DO in culturing tanks (p>0.05). According to the results of this study, the main source of nitrogen and phosphorus input to the tanks during a 45- day culturing period was from feeds. Shrimp and algal densities significantly affect the concentration of total ammonia, nitrite, nitrate and phosphate in water and an increase in shrimp density led to an increase of these compounds whereas, increasing the algal density led to the reduction of these compounds. Results indicated that increasing the density of *G.corticata* in all treatments, led to an increase in biomass of harvested shrimp and the co-culture of *G. corticata* with *L.vannamei* decreased the amount of nitrogen and phosphorus in both water and sediments and improved the water quality of *L.vannamei* culture.

Keywords: Nitrogen budget, Phosphorous budget, *Litopenaeus vannamei*, *Gracilaria corticata*, Zero water exchange

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Introduction

Semi-intensive and intensive shrimp farming in Iran has increased in recent years. Shrimp cultivation started in 1992 in Iran with the shrimp *Penaeus indicus;* however, as a result of economic losses of white spot syndrome (WSS), the Iranian Fisheries Organization decided to substitute it with the white leg shrimp *Litopenaeus vannamei*. This new species has produced good results in Iran, Major shrimp farming sites in Iran are located in the southern provinces including: Sistan and Baluchestan (500 ha), Bushehr (1500 ha), Hormozgan (1000 ha) and Khuzestan (300 ha) with a total production of 7900 tons (Baghaei and Sudagar, 2013; Kalbassi *et al*., 2013). Different stocking densities (20, 30 and 50 PL per m^2) have been tested to introduce *L. vannamei* to Iran, and the results indicated that shrimp production increased linearly with an increase in stocking density (Afsharnasab *et al.*, 2008). Some shrimp farmers in Iran tend to use a high stocking density of shrimp in their farms (Sareban *et al*., 2012). Regardless of the shrimp culture expansion in almost every coastal province of Iran, there is no data on the content of nutrients that are released into the environment by the effluents. Chemical budgets of semi-intensive and intensive culture systems of commercial shrimp ponds in Iran are unknown.

Mariculture activities generate huge quantities of inorganic wastes in the form of uneaten food and excretory products, out of which, more than 70% of them are released into the natural environment (Porter *et al*., 1987). Most of the food which is eaten by shrimp is excreted as metabolic waste which increases the inorganic nutrients and organic matter contents in the water and sediment (Attasat *et al.*, 2013).

Uneaten food which is released into the natural environment leads to eutrophication of the environment (Neori *et al*., 1991) and acute toxicity to the aquatic animals (Troell *et al.*, 1999; Neori *et al.*, 2000). In more densely stocked shrimp (*P. monodon*) ponds in Thailand and Alabama the major source of nitrogen (76–92%) and phosphorus (51–89%) was from feeds (Briggs and Funge-Smith, 1994; Couch, 1998). In shrimp (*L. vannamei*) ponds located in Tai lake region of China feeds contributed to an average of 61.24% and 81.01% of the total nitrogen and phosphorous gain, respectively in *P. vannanmei* ponds (Xia *et al*., 2004). In an integrated culture of shrimp (*L. vannamei*) and tomato (*Lycopersicon esculentum*) in low salinity ground water, most of the N (43.6%) and P (98.8%) entered to the system as shrimp food (Mariscal-Lagarda and Páez-Osuna, 2014).

The traditional method for maintaining pond water quality has been frequent water exchange, but this leads to high pollution of the receiving water. The environmental impacts of untreated effluents have raised concerns about the sustainability of shrimp farming. The reduction in the rate of water exchange has been studied all over the world to restrict the export of waste and to decrease its impact on coastal environments (Hopkins *et al*., 1995; Sandifer and Hopkins, 1996 ; fourooghifard *et al.*, 2017). An economical and feasible method that has been studied in recent years is the use of macro-algae to eliminate ammonium and nitrogen to

maintain good water quality (Neori *et al*., 1991; Buschmann *et al*., 1996b; Neori *et al*., 2004; Seema and Jayasankar, 2005 ; Rabiei *et al.*, 2014 ; Rabiei *et al.*, 2016).

Seaweeds can be utilized to eliminate the soluble part of this effluent. Utilizing seaweed *Gracilaria* co-cultivated with salmon in a tank system, as biofilter, in intensive mariculture systems demonstrated a notable increase in production rates and capability of removing 50% of the dissolved ammonium released by the fish in winter (Troell *et al.*, 1999). The productivity of *G. chilensis* near salmon farms and its nitrogen removal and photosynthetic performance has been investigated (Abreu *et al*., 2009). The biofiltering ability of *G. manilaensisi* has been tested to decrease inorganic –N waste of shrimp culture. Results have shown that co-culture of *G. manilaensis* and shrimp can enhance water quality and decrease waste water pollution released from the shrimp culture (Shukri and Surif, 2011). An integrated aquaculture system, based on shrimp-fish-seaweed, demonstrated that about 24% of the original nitrogen was retained in the form of aquaculture biomass, i.e. 15, 6 and 3% for shrimp, fish and seaweed respectively (Attasat *et al*., 2013). Nutrient uptake and macro algal growth performance have been investigated in short term (7–18 days) experiments in integrated aquaculture of the Pacific white shrimp, *L. vannamei*, and the macroalgae *G. tikvahia*, a rudimentary nutrient budget recovery of nearly 35% of the nitrogen input by shrimp and algal biomass was retained (Samocha *et al*., 2015).

Seaweeds are traditionally consumed in different part of the world. In Asian countries, seaweeds are often consumed as marine vegetables. The main uses of red seaweeds are as food and as sources of agar and carrageenan. Most agar are extracted from species of *Gelidium* and *Gracilaria* (McHugh, 2003). The red algae *G. corticata* is the main raw material for providing agar in Iran where agar industry has a noticeable capacity for growth. However, this industry is facing a shortage of raw materials. (Akbari *et al*., 2004; Foroughifard *et al*., 2005). The objective of this study was to investigate the partial nitrogen and phosphorous budgets for culturing of *L.vannamei* integrated with *G.corticata* under different densities of shrimp and algae in a zero water exchange system.

Materials and methods

Experimental design

The present study was carried out for a period of 45 days from August to October 2013 based on the culturing period (6- 7 weeks) of *G. corticata* in Iran (Akbari *et al*., 2004; Abkenar, 2007), at the Persian Gulf and Oman Sea Ecology Research Institute (PGOSERI). A two by three factorial was designed with two levels of shrimp density (S_1 and S_2 with 25 and 50 shrimp per m^2 respectively according to stocking density of shrimp in some farms in Iran) as one factor and three levels of red algae density (A_1, A_2 and A_3 with 0, 200 and 400g seaweed per m^2 respectively) as the second factor. Treatments are abbreviated as S_1A_1, S_1A_2, S_1A_3 (25 shrimp with 0, 200 and 400g algae per $m^{2)}$ and S_2A_1, S_2A_2, S_2A_3 (50 shrimp with 0, 200 and 400g algae per m^2). Experiments were carried out in triplicate in 18 round polyethylene tanks (1m^2 area). Tanks were

filled with 750 L of filtered sea water. A 40W compact fluorescent lamp was hung over each tank to provide sufficient light for growth of algae. A neutral photoperiod was used which was 12 hours light followed by 12 hours of darkness (12:12, L:D) (Yarish *et al*., 2012). Each tank was aerated by two pieces of 1" cylinder air stone with the aeration power of 5 $Lmin^{-1}$.

Culturing of shrimp and seaweed

The red algae *G. corticata* was obtained from coastal areas of Bandar-e Lengeh (26°33′29″N 54°52′50″E) Iran. Filtered seawater was utilized to wash the sea weed in the laboratory. The water was drained and the material was carefully inspected to remove encrusted organisms. *G. corticata* was cultured on a net tied to a round poly ethylene frame. Each net unit has about 50 mesh intersections. *Gracilaria* seedlings were tied at these places utilizing soft plastic thread (Juanich, 1988). All frames had 3 pods to hold nets 20 cm above the bottom. *Gracilaria* was separated in 10 g seedlings, twenty seedlings were fastened to each net for the treatment of 200g algae density and forty seedlings for the treatment of 400g algae density. Juvenile *L. vanamei* (5.82±0.11 g) were acquired from a shrimp farm located in Tiab shrimp farming site, Iran. Length and weight of shrimp were measured and their biomass was calculated before stocking in the tanks. Shrimp were stocked at a density of 25 and 50 shrimp per m^2 and were fed local commercial shrimp feed (Hormoz dam Shrimp grower Feed No. 2, containing 5.6 % nitrogen and 0.78% phosphorous), four times daily (06:00, 12:00, 18:00 and 22:00 h), which was dispersed directly to each tank. There was no water exchange, no fertilizer was used and no food or feces was eliminated from the treatment tanks during the culturing period.

Measurements

Water temperature, dissolved oxygen concentration (DO), pH and salinity (at 20 cm below the water surface) were measured every 3rd day during the culturing period. Water temperature and dissolve oxygen were measured using a portable meter (WTW, OXI 330i) with precisions of 0.1 °C and 0.1 mg L^{-1} respectively. Water pH was measured with a portable pH meter (WTW, pH 330i) with a precision of 0.01 pH unit. Salinity was measured by an Atago Hand Refractometer (model: S/Mill-E) with a precision of 0.5 PPT. Water samples for nutrient analysis were collected on a weekly basis from each tank and were immediately filtered utilizing Sartorius membrane filters (0.45 μm pore size). Samples were stored in a refrigerator until they were analyzed. Total ammonia, nitrite, nitrate and phosphate, were measured by spectrophotometric methods (Cecil 3041 Spectrophotometer) with a precision of $1 \mu g L^{-1}$ (Strickland and Parsons, 1972). At the end of the culturing period, all shrimp were harvested and weighted separately and stored in a freezer. Harvested algae were weighed with precisions of 1g and dried in an oven (at 70ºC for 48 h)(Motsara and Roy, 2008) and were kept in a freezer. Sediments were collected from each tank by siphoning, weighed and dried in an oven (at 70º C for 48 h) and were kept in a freezer until they were analyzed. Total nitrogen in shrimp, sea weed tissue and

sediments was measured by Kjeldahl method with a precision of 0.01g N in 1g of samples (SEAFDEC, 2001). Phosphorous in shrimp, sea weed tissue and sediments was measured using the spectrophotometric vanadium phosphomolybdate method with a precision of 1µg in 1g of samples (Motsara and Roy, 2008).

Shrimp, red algae and feed which were used for experiments, contained 3.07 0.32 and 5.65% nitrogen and 0.22, 0.03 and 0.78% phosphorous, respectively. Total N and P in sediments were calculated as follows: Total content of N and P in sediments=concentration of N and P in 1g sediment × total mass of sediments. Total amount of nitrogen and phosphorus (N and P) that entered into the system were calculated based on the amounts of N and P in water, shrimp and sea weed biomass on the first day of stocking and amount of food supplied during the culture period. Total amount of nitrogen and phosphorus (N and P) uptake and accumulation were calculated based on harvested shrimp biomass, harvested sea weed, and solute in water and sediment, at the end of culturing period,. The following formulae were used to compute the growth parameters of shrimp and seaweed (Ricker, 1975; Árnason *et al*., 2009). Specific growth rate (SGR) (% per day) =100 $(lnW2 - lnW_1) / (t_2 - t_1)$, where W_1 and W_2 are the weights of the shrimp and seaweed measured at times t_1 and t_2. Weight gained (WG) (%)=$100(W_2 - W_1)/W_1$, where W_1 and W_2 are the initial biomass and final biomass respectively. Feed conversion ratio (FCR)=Total feed intake/total weight gain of the shrimp during the growth period

Statistical analysis

All data were analyzed using SPSS 22.0 software by two way analysis of variance (ANOVA), with densities of shrimp and algae as fixed factors and shrimp production and growth parameters, temperature, oxygen, pH, in the morning and in the afternoon and nitrogen and phosphorus data as dependent variables. Significant differences between the treatment means were compared by Duncan's test. Differences were considered significant at $p<0.05$.

Results

Water physicochemical parameters

During the culturing period of *L. vannamei* with red algae *G. corticata*, water temperature ranged from 30.4 to 35.8 °C in the morning and from 30.4 to 35.8° C in the afternoon, with no significant differences between water temperatures in the morning and in the afternoon. DO ranged from 5.1 to 6.36 mgL^{-1} in the morning and from 5.63 to 6.56 mgL^{-1} in the afternoon, The maximum and minimum fluctuations of pH (7.9- 8.3 and 7.3-8.7) were found in treatments S2A3 and S1A3, respectively. No significant differences were observed between water temperature and pH in the morning and in the afternoon. Significant differences were observed between pH and DO in various treatments. Shrimp density had a significant effect on pH and DO in the morning and in the afternoon. The algae density didn't have a significant effect on pH and DO in culturing tanks (Table 1).

Table 1: Effects of shrimp and algal density on production, growth parameters of *Litopenaeus vannamei*, FCR and water quality in zero water exchange system during a 45- day culture period (Mean ± SE).

Variable	25shrimp per m²			50 shrimp per m²			Significance [a]		
	S_1A_1	S_1A_2	S_1A_3	S_2A_1	S_2A_2	S_2A_3	SD	AD	SD× AD
Shrimp:									
Final weight (g per shrimp) (*n=60*)	12.6 ±0.2	13.4 ± 0.2	14.1 ± 0.2	12.5 ± 0.2	13.2 ± 0.2	13.4 ± 0.2	**	**	**
Weight gained (%)	76.52 ± 8.2	102.1 ± 3.4	129.9 ± 2.9	10.10 ± 3.1	51.6 ± 5.7	64.3 ± 5.5	**	**	**
SGR(%day-1) (*n=3*)	1.72 ± 0.02	1.85 ±0.02	1.97 ± 0.0	1.7 ± 0.01	1.83 ± 0.02	1.85 ± 0.0	**	**	**
Survival rate (*n=3*)	81.3 ± 3.5	88.0± 2.3	94.7± 1.3	51.3 ± 1.3	64.7 ± 2.7	71.3 ± 2.4	**	**	NS
FCR	2.89 ± 0.3	2.12 ± 0.07	1.67 ± 0.04	25.02± 5.9	4.31 ± .45	3.42 ± 0.28	**	**	**
Algae :									
Weight gained (%)		73.67 ± 5.5	64.83 ± 2.1		23.50 ± 3.01	14.92 ± 1.9	**	**	**
SGR(%day-1) (n=3)	-	1.23 ± 0.07	1.11 ± 0.03	-	0.47 ± 0.05	0.31 ± 0.04	**	NS	NS
Water quality variables:									
Temperature (°C) at 6 a.m. (*n=45*)[b]	30.4-35.8	30.4 - 35.8	30.5-35.8	30.5-35.8	30.5-35.8	30.7-35.8	NS	NS	NS
Temperature (°C) at 4 p.m. (*n=45*)[b]	30.7 - 35.8	30.6 -35.8	30.7 -35.8	30.8-35.8	30.8-35.8	30.7-35.8	NS	NS	NS
pH at 6 a.m. (*n=45*)[b]	7.84 - 8.32	7.93- 8.32	7.93 -8.30	7.23-8.08	7.40-8.09	7.36-8.10	**	NS	NS
pH at 4 p.m. (*n=45*)[b]	7.82 - 8.31	7.91- 8.31	7.92 - 8.30	7.18-8.08	7.33-8.13	7.29-8.70	**	NS	NS
DO (mg L^{-1}) at 6 a.m. (*n=45*)[b]	5.41 - 6.29	5.42- 6.29	5.42 - 6.36	5.10- 6.31	5.28- 6.22	5.29-6.29	**	NS	NS
DO (mg L^{-1}) at 4 p.m. (*n=45*)[b]	5.76 - 6.56	5.79-6.49	5.73 - 6.48	5.85-6.44	5.67-6.43	5.63-6.56	**	NS	NS
Salinity (PPT) (*n=45*)[b]	37.00 - 43.50	37.0 - 43.5	37.00 - 43.50	37.00- 43.50	37.00- 43.50	37.00- 43.50	NS	NS	NS
Total Ammonium (µg L^{-1}) (*n=3*)[c]	201.0 ± 6.81	39.5 ±3.4	27.7 ±2.9	388.3 ±19.6	231.0± 9.2	190.0±4.9	**	**	NS
Nitrite (µg L^{-1})[c] (*n=3*)[c]	6545.3 ±154.8	5153.3±31.8	4230.7 ±137.4	11822.3±305.7	9057.7±49.1	7615.0 ±247.2	**	**	**
Nitrate (µg L^{-1})[c](*n=3*)[c]	57321.3±2551.4	48980.0 ±565.0	44236.0±748.9	92437.7±2894.9	86029.0 ± 1397.1	78951.3±2207.8	**	**	NS
Phosphate (µg L^{-1})[c](*n=3*)[c]	3381.7±158.1	2894.0 ± 28.6	2437.3±24.2	6106.7±156.8	5204.3±57.2	4370.7±87.8	**	**	**

[a] Results from two-way ANOVA ; SD = Shrimp Density; AD = Algae Density; SD × AD = Shrimp Density × Algae Density interaction
[b] During the culturing period
[c] End of culturing period

Concentration of total ammonia nitrite, nitrate and phosphate in water

The total concentration of ammonia initially increased in the first week but it gradually reduced in all treatments, during the study period (Fig. 1). The concentrations of nitrite, nitrate and phosphate gradually increased during the study period (Figs. 1, 2) There was a significant difference between concentration of nitrite, nitrate and phosphate in all treatments (p<0.05). Results demonstrated a significant positive correlation between shrimp densities and concentrations of total Ammonia, nitrite, nitrate and phosphate in tanks water (p<0.01). A significant negative correlation was found between algal densities and concentrations of total ammonia and nitrite (p<0.05) (Table 2).

Shrimp and algal densities significantly affected the concentration of total ammonia, nitrite, nitrate and phosphate in water (p<0.05). An increase in shrimp density led to an increase of these compounds, whereas increasing the algae density led to a reduction of these compounds (Table 1). The maximum and minimum concentrations of total ammonia (0.24±0.0 and 0.0 g m^{-2}), nitrite (7.95±0.2 and 2.8±0.1 g m^2), nitrate (62.3±1.9 and 29.8±0.5 g m^2) and phosphate (4.1±0.11 and 1.6±0.02 g m^{-2}) per culture area were found in treatments S2A1 and S1A3, respectively (Table 3).

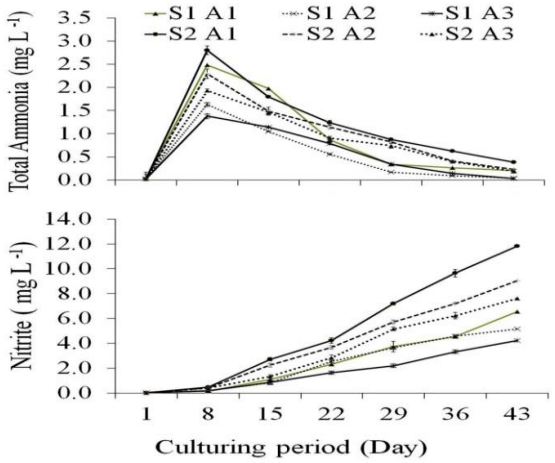

Figure 1: Concentration of total ammonia and nitrite, in treatments during the integrated culture period of *Litopenaeues vannamei* with *Gracilaria corticata* (Mean ± SE).

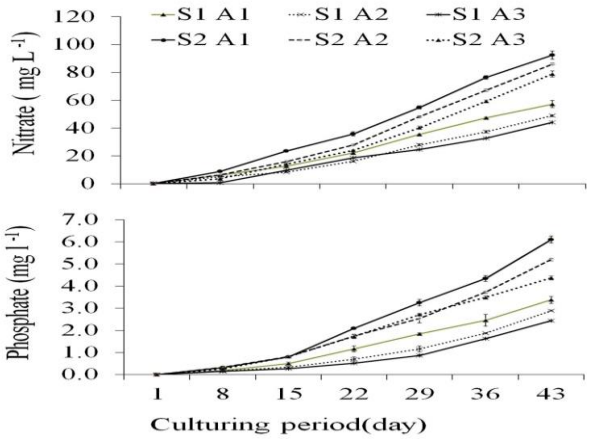

Figure 2: Concentration of total nitrate and phosphate in treatments during the integrated culture period of *Litopenaeues vannamei* with *Gracilaria corticata* (Mean ± SE).

Table 2: Correlation between shrimp and algal densities and total ammonia, nitrite, nitrate and phosphate in zero water exchange system during a 45- day culture period of *Litopenaeues vannamei* with *Gracilaria corticata.*

		Correlations			
		Total Ammonia	Nitrite	Nitrate	phosphate
Shrimp Density	Pearson Correlation	.734**	.826**	.946**	.892**
	Sig.	.001	.000	.000	.000
Algae Density	Pearson Correlation	-.617**	-.525*	-.288	-.420
	Sig.	.006	.025	.246	.083

**. Correlation is significant at the 0.01 level

*. Correlation is significant at the 0.05 level (*n= 18*)

Table 3: Total amounts of input and output of ammonia, nitrite, nitrate and phosphate (g m^{-2}) for integrated culture of *Litopenaeues vannamei* with red sea algae *Gracilaria corticata* in a zero water exchange system during a 45- day trial (Mean ± SE, *n*=3).

	Total Ammonia			NO2			NO3			Phosphate		
Treatment	Input	Output	Net Load	Input	Output	Net Load	Input	Output	Net Load	Input	Output	Net Load
S1 A1	0.02 ±0.0	0.14 ±0.0	0.12 ±0.0	0.03 ± 0.0	4.4 ± 0.10	4.4 ± 0.1	0.1 ± 0.0	38.7 ± 0 .7	38.6 ±1.7	0.0 ±0.0	2.28 ±0.11	2.3 ± 0.1
S1 A2	0.02 ±0 .0	0.03 ±0.0	0.01 ±0.0	0.03 ± 0.0	3.5 ±0.02	3.4 ± 0.0	0.1± 0.0	33.1 ± 0.38	33 ±0.38	0.0 ± 0.0	1.95 ± 0.02	1.9 ± 0.02
S1 A3	0.02 ± 0.0	0.02 ±0.0	0.0 ± 0.0	0.03 ± 0.0	2.86 ±0.09	2.8 ± 0.1	0.1± 0.0	29.9 ± 0.51	29.8 ±0.5	0.0 ± 0.0	1.65 ± 0.02	1.6 ± 0.02
S2 A1	0.02 ± 0.0	0.26 ±0.1	0.24 ± 0.0	0.03 ± 0.0	7.98 ±0.2	7.95 ± 0.2	0.1± 0.0	62.4 ±1.95	62.3 ±1.9	0.0 ± 0.0	4.12 ± 0.11	4.1 ± 0.11
S2 A2	0.02 ± 0.0	0.16 ±0.0	0.14 ± 0.0	0.03 ± 0.0	6.1± 0.03	6.1 ± 0.0	0.1± 0.0	58.1 ± 0.94	58 ± 0.94	0.0 ± 0.0	3.51 ± 0.04	3.5 ± 0.04
S2 A3	0.02 ±0.0	0.13 ±0.0	0.11± 0.0	0.03 ± 0.0	5.14 ±0.17	5.1 ± 0.2	0.1± 0.0	53.3 ± 1.49	53.2 ±1.5	0.0 ± 0.0	2.95 ± 0.06	2.9 ± 0.06

Shrimp and seaweed growth and productions

The maximum and minimum SGR (1.97±0.0 and 1.7±0.01 %day^{-1}), survival rate (94.7±1.3 and 51.3±1.3 %) and weight gained (129.9±2.9 and 10.10±3.1%) of *L. vanamei* were found in treatments S_1A_3 and S_2A_1, respectively. The maximum and minimum SGR (1.23±0.07 and 0.31±0.04 % day^{-1}) and weight gained (73.67±5.5 and 14.92±1.9 %) of *G. corticata* were related to the treatments S_1A_2 and S_2 A_3, respectively. Results indicated that shrimp density significantly affected the final weight, weight gain, SGR and survival rate of *L.vaname*. It was also observed that

shrimp density significantly affected weight gain and SGR of *G. corticata*. A significant interaction between shrimp and algal density was observed on growth parameters of *L.vanamei* and *G. corticata*, during the 45- day culture period (Table 1).

Nitrogen budget

The main source of nitrogen input to the tanks during the 45- day culture period was from feeds (17.79 gm^{-2} in treatments with 25 shrimp m^{-2} and 35.59 gm^{-2} in treatments with 50 shrimp m^{-2}). Partial nitrogen input provided by shrimp food in treatments was 79.8±0.0, 77.5±0.0, 75.4±0.0, 79.8±0.0, 78.7±0.0, and 77.6±0.0% for treatments S_1A_1, S_1A_2, S_1A_3, S_2A_1, S_2A_2 and S_2A_3, respectively (Table 4). The maximum and minimum concentrations of nitrogen in water (16.7±0.4 and 7.63±0. m^{-1}) were found in treatments S2A1 and S_1A_3, respectively. The maximum and minimum contents of nitrogen in sediments (4.46±0.06 and 1.3±0.02 g m^{-1}) were found in treatments S2A1 and S_1A_2, respectively. At the end of the culture period, the partial nitrogen in shrimp biomass was 35.1±0.9, 39.6±0.3, 41.9±0.5, 21.7±1.6, 28.0±1.5, and 31.0±1.3% in treatments S_1A_1, S_1A_2, S_1A_3, S_2A_1, S_2A_2 and S_2A_3, respectively. The partial nitrogen in seaweed biomass was 0.0, 4.7±1.0, 9.4±0.6, 0.0, 1.8±1.3 and 3.4±0.6% in treatments S_1A_1, S_1A_2, S_1A_3, S_2A_1, S_2A_2 and S_2A_3, respectively. There were significant differences between total input and total output nitrogen in all treatments (p<0.05), the difference between input and output nitrogen may have been released into the atmosphere (Table 4).

Table 4: Nitrogen budget for different treatments of integrated culturing of *Litopenaeues vannamei* and *Gracilaria corticata* in a zero water exchange system during a 45- day trial (Mean ± SE, *n*=3).

Treatment	Nitrogen Input					Nitrogen Output					
	Feed	Shrimp	Algae	Water	Total	Shrimp	Algae	Sediments	Water	Total	Unaccounted (lost)
$S_1 A_1$ (g m^{-2})	17.8 ± 0.0	4.5 ± 0.0	0.0 ± 0.0	0.05 ± 0.0	22 .3 ± 0.0	7.8 ± 0.3	0.00 ± 0.0	1.74 ± 0.0	10.2 ± 0.4	19.8 ± 0.4	2.5 ± 0.4
Kg ha^{-1}	178 ± 0.0	45 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	22 3 ± 0.0	78 ± 3	0.00 ± 0.0	17.4 ± 0.0	102 ± 4	198 ± 0.4	25 ± 4
%	79.8 ± 0.0	20.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	100	35.1 ± 0.9	0.0±0.0	7.8 ± 0.1	45.7 ± 0.9	88.6 ± 0.4	11.4 ± 3.3
$S_1 A_2$(g m^{-2})	17.8 ± 0.0	4.5 ± 0.0	0.65 ± 0.0	0.05 ± 0.0	23 ± 0.0	9.1 ± 0.1	1.1 ± 0.05	1.3 ± 0.02	8.5 ± 0.1	20.0 ± 0.3	2.9 ± 0.3
Kg ha^{-1}	178 ± 0.0	45 ± 0.0	6.5 ± 0.0	0.5 ± 0.0	230 ± 0.0	91 ± 1	11 ± 0.5	13 ± 0.2	85 ± 1	20.0 ± 3	29 ± 3
%	77.5 ± 0.0	19.5 ± 0.0	2.8 ± 0.0	0.2 ± 0.0	100	39.6 ± 0.3	4.7 ± 1.0	5.7 ± 0.4	37.2 ± 0.2	87.3 ± 0.3	12.7 ± 2.0
$S_1 A_3$(g m^{-2})	17.8 ± 0.0	4.5 ± 0.0	1.3 ± 0.0	0.05 ± 0.0	23.6 ± 0.0	9.9 ± 0.2	2.2 ± 0.05	1.69 ± 0.03	7.63 ± 0.1	21.4 ± 0.7	2.2 ± 0.2
Kg ha^{-1}	178 ± 0.0	45 ± 0.0	13.1 ± 0.0	0.5 ± 0.0	236 ± 0.0	99 ± 2	22 ± 0.5	16.9 ± 0.03	76.3 ± 1	214 ± 7	22 ± 2
%	75.4 ± 0.0	18.9 ± 0.0	5.5 ± 0.0	0.2 ± 0.0	100	41.9 ± 0.5	9.4 ± 0.6	7.2 ± 0.5	32.3 ± 0.3	90.8 ± 0.2	9.2 ± 1.7
$S_2 A_1$(g m^{-2})	35.8 ± 0.0	8.9 ± 0.0	0.0 ± 0.0	0.05 ± 0.0	44.6 ± 0.0	9.7 ± 0.3	0.0 ± 0.00	4.46 ± 0.06	16.7 ± 0.4	30.9 ± 0.7	13.7 ± 0.7
Kg ha^{-1}	358 ± 0.0	89 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	446 ± 0.0	97 ± 3	0.0 ± 0.00	44.6 ± 0.06	167 ± 4	309 ± 7	137 ±7
%	79.8± 0.0	20.1± 0.0	0.0 ± 0.0	0.1± 0.0	100	21.7 ± 1.6	0.0 ± 0.0	10.0 ± 0.6	37.5 ± 1.1	69.2 ± 1.0	30.8 ± 2.3
$S_2 A_2$(g m^{-2})	35.8 ± 0.0	8.9 ± 0.0	0.65 ± 0.0	0.05 ± 0.0	45.2 ± 0.0	12.7 ± 0.4	0.8 ± 0.02	4.17 ± 0.09	15.10 ± 0.2	32.7 ± 0.2	12.4 ± 0.2
Kg ha^{-1}	358 ± 0.0	89 ± 0.0	6.5 ± 0.0	0.5 ± 0.0	452 ± 0.0	127 ± 0.4	8 ± 0.2	41.7 ± 0.9	151 ± 2	327 ± 2	124 ±2
%	78.7± 0.0	19.7± 0.0	1.5 ± 0.0	0.1± 0.0	100	28.0 ± 1.5	1.8 ± 1.3	9.2 ± 1.0	33.4 ± 0.6	72.5 ± 0.3	27.5 ± 0.8
$S_2 A_3$(g m^{-2})	35.8 ± 0.0	8.9 ± 0.0	1.31 ± 0.0	0.05 ± 0.0	45.9 ± 0.0	14.2 ± 0.4	1.6 ± 0.02	3.88 ± 0.06	13.7 ± 0.3	33.4 ± 0.1	12.5 ± 0.1
Kg ha^{-1}	358 ± 0.0	89 ± 0.0	13.1 ± 0.0	0.5 ± 0.0	459 ± 0.0	142 ± 0.4	16 ± 0.02	38.8 ± 0.6	137 ± 3	334 ± 1	125 ± 1
%	77.6 ± 0.0	19.4 ± 0.0	2.9 ± 0.0	0.1± 0.0	100	31.0 ± 1.3	3.4 ± 0.6	8.5 ± 0.8	29.9 ± 1.1	72.7 ± 0.2	27.2 ± 0.4

Phosphorus budget

The main source of phosphorus input to the tanks during the 45- day culture period was from shrimps feed (2.47 gm^{-2} in treatments with 25 shrimp m^{-2} and 4.93gm^{-2} in treatments with 50 shrimp m^{-2}). Partial input of phosphorus provided by shrimp food, in treatments was 88.6, 86.5, 84.5, 88.6, 87.6, and 86.5% for treatments S_1A_1, S_1A_2, S_1A_3, S_2A_1, S_2A_2 and S_2A_3, respectively (Fig. 2). At the end of culture period, the maximum content of the phosphorus input was found in sediments in all treatments. The contents of phosphorus in sediments were 52.8±1.8, 46.8±0.3, 42.7±0.9, 64.4±0.7, 61.6±0.7 and 61.6±1.1% in treatments S1A1, S_1A_2, S_1A_3, S_2A_1, S_2A_2 and S_2A_3, respectively (Table 5). There was no significant difference between total phosphorus input and output in treatments (p>0.05).

Table 5: Phosphorus budget for integrated culturing of *Litopenaeues vannamei* and *Gracilaria corticata* in a zero water exchange system during a 45-day trial (Mean ± SE, *n*=3).

Treatment	Phosphorus Input					Phosphorus Output					
	Feed	Shrimp	Algae	Water	Total	Shrimp	Algae	Sediments	Water	Total	Unaccounted (lost)
S_1 A_1 (g m^{-2})	2.5 ± 0.0	0.3 ± 0.0	0.0± 0.0	0.0 ± 0.0	2.78 ± 0.0	0.5 ± 0.02	0.0 ± 0.0	1.5 ± 0.1	0.7 ± 0.06	2.7 ± 0.0	0.05 ±0.01
Kg ha^{-1}	25 ± 0.0	3 ± 0.0	0.0± 0.0	0.0 ± 0.0	27.8 ± 0.0	5 ± 0.2	0.0 ± 0.0	15 ± 0.1	7 ± 0.6	27 ± 0.0	0.5 ±0.1
%	88.6 ± 0.0	11.3± 0.0	0.0± 0.0	0.0± 0.0	100	18.7 ± 0.9	0.0 ± 0.0	52.8 ± 1.8	26.7± 1.2	98.3 ± 0.3	1.7 ± 0.3
S_1 A_2(g m^{-2})	2.5 ± 0.0	0.3 ± 0.0	0.07 ± 0.0	0.0 ± 0.0	2.85 ± 0.0	0.6 ± 0.02	0.24 ± 0.0	1.4 ± 0.1	0.6 ± 0.0	2.8 ± 0.0	0.04±0.02
Kg ha^{-1}	25 ± 0.0	3 ± 0.0	0.7 ± 0.0	0.0 ± 0.0	28.5 ± 0.0	6 ± 0.2	2.4 ± 0.0	14 ± 1	6 ± 0.0	28 ± 0.0	0.4±0.2
%	86.5± 0.0	11.1± 0.0	2.4± 0.0	0.0± 0.0	100	21.7 ± 0.6	8.4 ± 0.3	46.8 ± 0.3	22.3 ± 0.2	98.7 ±0.5	1.3± 0.5
S_1 A_3(g m^{-2})	2.5 ± 0.0	0.3 ± 0.0	0.14 ± 0.0	0.0 ± 0.0	2.9 ± 0.0	0.7 ± 0.0	0.43 ± 0.0	1.3 ± 0.0	0.5 ± 0.0	2.9 ± 0.0	0.04±0.01
Kg ha^{-1}	25 ± 0.0	3 ± 0.0	1.4 ± 0.0	0.0 ± 0.0	29 ± 0.0	7 ± 0.0	4.3 ± 0.0	13 ± 0.0	5 ± 0.0	29 ± 0.0	0.4±0.1
%	84.5± 0.0	10.8± 0.0	4.7± 0.0	0.0± 0.0	100	22.8 ± 0.1	14.6 ± 0.6	42.7 ± 0.9	18.4 ± 0.2	98.5± 0.3	1.5±0.3
S_2 A_1(g m^{-2})	4.9 ± 0.0	0.6 ± 0.0	0.0± 0.0	0.0 ± 0.0	5.57 ± 0.0	0.6 ± 0.02	0.0 ± 0.0	3.6 ± 0.04	1.3 ± 0.03	5.5 ± 0.0	0.02±0.02
Kg ha^{-1}	49 ± 0.0	6 ± 0.0	0.0± 0.0	0.0 ± 0.0	55.7 ± 0.0	6 ± 0.2	0.0 ± 0.0	36 ± 0.4	13 ± 0.3	55 ± 0.0	0. 2±0.2
%	88.6± 0.0	11.4± 0.0	0.0± 0.0	0.0± 0.0	100	11.3± 0.3	0.0 ± 0.0	64.4 ± 0.7	23.8 ± 0.3	99.5 ± 0.3	0.4±0.3
S_2 A_2(g m^{-2})	4.9 ± 0.0	0.6 ± 0.0	0.07 ± 0.0	0.0 ± 0.0	5.63 ± 0.0	0.9 ± 0.04	0.2 ± 0.00	3.5 ± 0.1	1.2 ± 0.01	5.6 ± 0.01	0.01±0.0
Kg ha^{-1}	49 ± 0.0	6 ± 0.0	0.7 ± 0.0	0.0 ± 0.0	56.3 ± 0.0	9 ± 0.04	2 ± 0.00	35 ± 1	12 ± 0.1	56 ± 0.1	0.1±0.0
%	87.6± 0.0	11.2± 0.0	1.2± 0.0	0.0± 0.0	100	15.6±.0.7	3.0±0.1	61.6 ± 0.7	19.7 ±0.2	99.9 ± 0.0	0.1±0.0
S_2 A_3(g m^{-2})	4.9 ± 0.0	0.6 ± 0.0	0.14 ± 0.0	0.0 ± 0.0	5.70 ± 0.0	0.9 ± 0.04	0.3 ± 0.01	3.6 ± 0.04	1.0 ± 0.02	5.6 ± 0.01	0.07±0.03
Kg ha^{-1}	49 ± 0.0	6 ± 0.0	1.4 ± 0.0	0.0 ± 0.0	57.0 ± 0.0	9 ± 0.4	3 ± 0.1	36 ± 0.4	10 ± 0.2	56 ± 0.1	0.7±0.3
%	86.6± 0.0	11.1± 0.0	2.4± 0.0	0.0± 0.0	100	16.4 ± 0.7	5.4 ± 0.1	61.6 ± 1.1	15.3±0.4	98.7 ±0.6	1.3±0.6

Discussion

The reduction in the rate of water exchange can be applied to restrict the export of waste and to decrease the impact on coastal environments. According to results of this study, choosing suitable stocking densities of red algae *G. corticata* and *L. vannamei* in a zero water exchange system led to improved water quality and increased algae and shrimp production. According to the results, the best performance of *L. vannmei* (maximum survival rate, SGR, WG and minimum FCR) was observed in treatment S_1A_3 (25 shrimp m^{-2} and 400g seaweed m^{-2}), while concentrations of total ammonia, nitrite and nitrate in this treatment were significantly lower than in the others. The highest concentrations of total ammonia (0.388 mg L^{-1}), nitrite (11.822 mg L^{-1}) and nitrate (92.437 mg L^{-1}) were observed in treatment S_2A_1 (50 shrimp m^{2-} without any seaweed). The "safety level" for rearing *L. vannamei* was estimated to be 3.95 mg l^{-1} for ammonia-N, 25.7 mg L^{-1} for nitrite -N and 177 mg L^{-1} for nitrate –N in 35‰ (Lin and Chen, 2001; Tsai and Chen, 2002; Lin and Chen, 2003). As the concentrations of nitrogen compounds in all treatments are below the "safety levels", it may be concluded that bad performance of *L. vannmei* (minimum

survival rate, SGR, WG and maximum FCR) in treatment S_2A_1 was not the result of nitrogen compounds. Some authors reported an inverse relationship between survival rate and growth parameters of *L. vannamei* and stocking density (Williams *et al*., 1996; Mude and Naik, 2014). In this study the minimum and maximum fluctuations in pH (7.9- 8.3 and 7.29-8.70) were found in S_1A_3 and S_2A_3, respectively. Results indicated that shrimp density significantly affects pH, DO, and load of nutrients in the water (Table 1). A bi-weekly monitoring of the inlet and outlet water of semi-intensive shrimp (*L. vannamei*) culture ponds revealed that the pH of the inlet and outlet water ranged between 7.7 and 8.5 (Casillas-Hernández *et al*., 2007). Another study on nutrient mass balances in intensive shrimp (*L. vannamei*) ponds indicated that water pH ranged between 7.8±0.2 and 8.0±0.3 at 7:30 and 14:00 h, respectively (Van Nguyen and Maeda, 2015).

In this study the main source of nitrogen was 75.4-79.8% for treatments with 25 shrimp per m^2 and 77.6±0.0 - 79.8± 0.0% for treatments with 50 shrimp per m^2. Feeds were the main source of phosphorous (84.5-8.6% for treatments with 25 shrimp per m^2 and 86.6±0.0-88.6±0.0% for treatment with 50 shrimp per m^2). About 0.1- 0.2 % of total nitrogen input and 0.0 % of phosphorous input were from water intake. Similar results were found in more densely stocked shrimp (*Penaeus monodon*) ponds in Thailand and Alabama where the major source of nitrogen (76–92%) and phosphorus (51–89%) was from feed (Briggs and Funge-Smith, 1994; Couch, 1998).

In shrimp (*L. vannamei*) ponds located in the Tai lake region of China, feeds contributed an average of 193.81 kg ha^{-1} (61.24%) and 45.20±2.12 kg ha^{-1} (81.01%) of the total nitrogen and phosphorous gain, respectively in *L. vannanmei* ponds. Water pumped into the ponds brought an average of 83.57 kg ha^{-1} (26.96%) and 8.48±0.57 kg ha^{-1} (15.20%) of the total nitrogen and phosphorous input, respectively (Xia *et al*., 2004). In a semi-intensive shrimp farm in North-Western Mexico, feed accounted for 76.0% and 83.4% of the nitrogen and phosphorus input, respectively (Páez-Osuna *et al*., 1997).

In an integrated culture of shrimp (*L. vannamei*) and tomato (*L. esculentum*) with low salinity ground water, most of the N (43.6%) and P (98.8%) entered the system as shrimp food (Mariscal-Lagarda & Páez-Osuna, 2014).

In contrast, in semi-intensive shrimp (*L. vannamei*) ponds in Honduras, water exchange accounted for the majority of nitrogen and phosphorus gained by the ponds; water intake contributed 63%, while feed contributed 36% of the nitrogen. On the other hand, the main phosphorus input was mostly from water (51%) and feed (47%) (Teichert-Coddington *et al*., 2000).

In this study, the shrimp production of *P. vannanmei* in the treatments with 25 shrimp per m^2 contained an average of 7.8±0.3, 9.1±0.1 and 9.9±0.2 g m^{-2} (equal to 78±3, 91±1 and 99±2 kg ha^{-1}) of total nitrogen input. The shrimp production in the treatments with 50 shrimp per m^2 (S_2A_1, S_2A_2 and S_2A_3), contained an average of 9.7±0.3, 12.7±0.4 and 14.2±0.4g m^{-2} (equal to 97±3, 127±4, and 142±4 kg ha^{-1}) of total nitrogen input. In

a 112-day culture period, in the Tai lake region of China, the shrimp product of *L. vannanmei* ponds contained an average of 102.81 kg ha^{-1} which was equal to 32.94% of the total nitrogen input (Xia *et al*., 2004).

In an integrated culture of shrimp (*L. vannamei*) and tomato (*L. esculentum*), in Mexico, during a 133-day period, about 15.2% of the N input, and 8.9% of the P input, were converted to harvested shrimp and about 13.4% of N input was unaccounted for, and was assumed to be lost to the atmosphere via de nitrification and volatilization (Mariscal-Lagarda and Páez-Osuna, 2014).

In contrast, in Thailand, budgets for nitrogen and phosphorus for a series of intensive shrimp ponds were determined over two or three culture cycles. Results indicated that shrimp (*P. monodon*) converted 24% of feed nitrogen and 13% of feed phosphorus to flesh (Briggs and Funge-Smith, 1994). In semi-intensive shrimp farms in Honduras, partial chemical budgets demonstrated that commercial ponds stocked with 7–10 shrimp m^{-2} retained 6.5% of the nitrogen and 31% of the phosphorus gained through feeds, fertilizer, and water (Teichert-Coddington *et al*., 2000).

In this study, as there was no water exchange, no significant difference was found between total input and total output phosphorous (Table 5). About 46.8±0.3 to 64.4±0.7% of total phosphorous input was found in sediments. In shrimp (*L. vannamei*) ponds in the Tai lake region of China, about 74.37% of total phosphorous input was found in sediments (Xia *et al*., 2004).

Despite the zero water exchange, during the culture period, there was a significant difference between total nitrogen input and total nitrogen gained by shrimp and algae, solute in water and accumulated in sediments. Nitrogen may be lost via denitrification or volatilization of ammonia (Teichert-Coddington *et al*., 2000). Denitrification involves the reduction of nitrate via nitrite and nitric oxide to nitrous oxide or nitrogen gas (Zumft, 1997).

In this study, about 9.2±1.7 - 30.8±2.3% of the total nitrogen input was lost through volatilization (Table 4). In comparison, in integrated culture of shrimp (*L.vannamei*) and tomato (*L. esculentum*) in Mexico, about 13.4 % of total nitrogen input was lost through evaporation (Mariscal-Lagarda and Páez-Osuna, 2014). In contrast in shrimp (*L. vannamei*) ponds in the Tai Lake region of China about 54.86% of the total nitrogen input was lost through volatilization, denitrification and deposition (Xia *et al*., 2004).

The results of this study demonstrated that increasing the density of *G. corticata* in all treatments, led to an increase in biomass of harvested shrimp (Table 1), consequently leading to an increase of nitrogen and phosphorus uptake by shrimp. The productivity of species in a mixed culture system depends on the growth performance of both species in the system (Qian *et al.*, 1996). In this study, the best growth rate of *G. corticata* was found in the treatment S1A2 and S1A3 (with 25 shrimp m^{-2}) (Table 1). Increase in density of shrimp can result in an increase in turbidity and a decrease in the intensity of light which can restrict the growth of algae. Integrated cultivation of the red alga

Kappuphycus alvarezii and the pearl oyster *Pinctada martensi* revealed a strong relationship between algal growth and uptake of nitrogenous wastes within the tested ranges, which proved that the algae can efficiently remove nitrogenous wastes when conditions (e.g. light, temperature, turbidity) are suitable for the high growth of algae (Qian *et al*., 1996).

From the results of this study, during the culture period, the maximum and minimum concentrations of nitrite, nitrate and phosphate were found in treatments S_2A_1 (high density of shrimp without any algae) and S_1A_3 (with low density of shrimp and high density of algae) respectively (Figs. 1, 2). Integrated cultivation of Salmonids and *G. chilensis* indicated that the development of *Gracilaria* using fish tank effluents permits a diversification of the production as a result of the biofiltering efficiency of *Gracilaria* in eliminating nitrogen and phosphorus (Buschmann *et al.*, 1996a).

The results demonstrated a significant reverse correlation between seaweed density and concentration of total ammonia, nitrite, and phosphate in water (Table 2). These results validate the accepted hypothesis that the seaweeds in integrated culture systems, convert the metabolic wastes of animals into algal biomass resulting in high growth rates (Harlin *et al*., 1979). It has been reported that the productivity of both *Gracilaria* spp. and crabs increased when they were cultivated in the same pond (Trono 1989). A study on the biofiltering ability of seaweeds in decreasing inorganic nitrogen of shrimp culture ponds revealed that *G. manilaensis* was able to reduce up to 83.65% of NH_4^+, 33.33% of NO_2^- and 68.42% of NO_3^- after 24 h., Based on this, it has been suggested that the co-culture of *G. manilaensis* together with shrimp is necessary in order to enhance water quality and to decrease waste pollution released from the shrimp (Shukri and Surif, 2011). In this study the maximum SGR (1.22±0.07% day^{-1}) of *G. corticata* was observed in treatment S_1A_2. Similarly, the growth rate of 1.11%/day was obtained for red seaweed *Kappaphycus alvarezii* in co-culture with the white leg shrimp *L. vannamei* in floating cages (Lombardi *et al*., 2006) The red algae *G. corticata* is the main raw material for providing agar in Iran where agar industry has a noticeable capacity for growth. However, this industry is facing a shortage of raw materials. (Akbari *et al.*, 2004; Foroughifard *et al*., 2005). This study demonstrated that co-culture of *L. vannamei* and *G. corticata* in a zero water exchange system, could enhance total production of *L. vanamei*, decrease the amount of nitrogen and phosphorus both in water and sediments and consequently improve the water quality. However, the increase in seaweed biomass could enhance the production of shrimp biomass and total nitrogen uptake by shrimp. In addition, increase in shrimp density could result in a decrease in production of shrimp and increase in turbidity which consequently would result in a decrease in production of *G.corticata*. From the results of the present study, a density of 400 g seaweed m^{-2} and 25 shrimp m^{-2} could be suitable for integrated culture of *L. vanamei* and *G. corticata* in a zero water exchange system.

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