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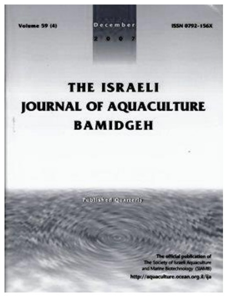

Evaluation of Crinkle Grass *Rhizoclonium riparium* in a Diet for Pacific White Shrimp *Penaeus vannamei* Post Larvae

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Keywords: green seaweed meal; mineral replacement; white shrimp post-larvae; feeding trial; soybean replacement.

Abstract

A feeding experiment to evaluate the inclusion of *Rhizoclonium* raw meal (RM) in diets for growth and feed efficiency in Pacific white shrimp, *Penaeus vannamei* was conducted. Five diets were formulated containing increasing levels of RM inclusion: 0%, (control), 5.25%, 10.50%, 15.75%, and a diet with 15.75% RM totally replacing the mineral mix. The diets were fed to shrimp post larvae for 60 days with 4 replicates. Survival rates were excellent (98-93%) and similar for all treatments. Final average body weight (FABW) was highest in shrimp fed the diet containing 5.25% RM, and lowest with the 10.50% RM diet; FABW of the other groups were either statistically similar or lower than the control group. A similar pattern was also observed for specific growth rate (SGR) and protein gain (PG). Food conversion efficiency (FCE) was highest in shrimp fed the diet containing 5.25% RM; protein efficiency ratio (PER) was higher in all groups fed diets containing RM than the control group. Replacing the dietary mineral component with RM did not significantly affect the growth, feed efficiency, and survival rate. The present study demonstrated that *Rhizoclonium riparium var. implexum* is a possible complementary protein source and could replace from 15%-45% imported soybean meal (SBM) in the *P. vannamei* post larvae diet. An additional 1% RM could replace the dietary minerals.

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Introduction

Several feeding trial studies in shrimp have shown that many types of macroalgae can be used to enhance growth, feed utilization, stress response, starvation tolerance, disease resistance, and carcass quality (Declarador, 2014; Santizo et al, 2014). Use of plant protein concentrate (PC) as a functional ingredient either for economic reasons or for enhancement of nutritional quality of the food product has gained considerable attention (Wong and Cheung, 2001).

Green algae such as *Rhizoclonium riparium* (Chlorophyceae) are a potential ingredient in aquafeeds although often considered a nuisance in fishponds as they remove dissolved oxygen and reduce space, resulting in hindered growth of cultured animals (Chao et al., 2004; Caffrey, 1992). *R. riparium* contains significant amounts of proteins, vitamins, and minerals (Chakraborty and Santra, 2008) and has potential for large scale culturing year-round (Chao et al., 2004). Little is known about the nutritional quality of *Rhizoclonium* meal in the diet of white shrimp and whether or not it could replace an imported and more expensive ingredient such as soybean meal.

The objective of this study was to evaluate the effect of *R. riparium var. implexum* as a feed ingredient and as partial replacement of soybean meal on growth and feed efficiency of Pacific white shrimp, *Penaeus vannamei*.

Materials and Methods

Seaweed collection and preparation. R. riparium var. implexum (Dillwyn) Kützing (1843), previously identified by Dr. Anicia Q. Hurtado – Ponce (Bunda et al 2015), was collected from brackish water ponds of the University of the Philippines Visayas (UPV) located in Leganes, Iloilo. The collected samples were transported to the University hatchery (about 50 km) in Miagao, Iloilo for processing.

Diet preparation. R. riparium samples were cleaned of debris and washed with freshwater. The samples were processed as described by Bunda (2015); they were air-dried for 48 h, subsequently oven dried for 24 h at 60°C, and pulverized using a mechanical grinder. The powdered *Rhizoclonium sp.* was stored at -20°C until use.

A control diet and test diets with different levels of inclusion of RM (0% (control), 5.25%, 10.50%, 15.75% and a test diet containing 15.75% RM simultaneously replacing minerals, were formulated following Bunda (2015) (Table 1). The diets were prepared at the Nutrition Laboratory of the Institute of Aquaculture, College of Fisheries and Ocean Sciences (CFOS-IA), University of the Philippines Visayas (UPV). Prior to mixing, all ingredients were passed through a 150μm sieve. All ingredients were mixed, pelleted, and subsequently oven-dried at 60°C for at least 8 h. The diets were crumbled to an appropriate size and stored at -20°C until use.

Rearing and feeding. Post larval shrimp from a commercial shrimp hatchery in Guimbal, Iloilo were nursed in a 1 ton capacity tank at the University of the Philippines Visayas Multi-Species Hatchery. The shrimp were acclimated and fed a commercial diet for 2 weeks. Prior to the experiment, the shrimp were randomly selected and screened for white spot syndrome virus (WSSV) by a one-step PCR; those used in the present study were free of the pathogen.

Table 1. Composition and proximate analysis of experimental diets fed to Pacific white shrimp (*Penaeus vannamei*) post-larvae for 60 days.

Ingredients	*Diet*				
	D1	*D2*	*D3*	*D4*	*D5*
	0	*5.25*	*10.5*	*15.75*	*15.75*
Danish fish meal	250.00	250.00	250.00	250.00	250.00
Squid meal	29.00	29.00	29.00	29.00	29.00
Soybean meal	350.00	297.50	245.00	192.50	192.50
Bread flour	117.00	117.00	117.00	117.00	117.00
Cod liver oil	63.00	63.00	63.00	63.00	63.00
Lecithin	5.00	5.00	5.00	5.00	5.00
Carboxymethylcellulose	130.50	130.50	130.50	130.50	130.50
Lignobond	15.00	15.00	15.00	15.00	15.00
Vitamin mix**	10.00	10.00	10.00	10.00	10.00
Mineral mix	10.00	10.00	10.00	10.00	0.00
Dicalphosphate	20.00	20.00	20.00	20.00	20.00
BHT	0.50	0.50	0.50	0.50	0.50
Rhizoclonium meal	0.00	52.50	105.00	157.50	167.50
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Analyzed Proximate Composition (dry matter basis, %)					
Moisture	2.26	6.39	3.14	5.41	6.03
Crude protein	38.4	38.6	35.4	34.8	34.1
Crude lipid	8.7	9.3	9.0	8.9	9.1
Ash	13.5	15.5	17.1	19.3	19.2
NFE	39.4	36.7	38.5	37.0	37.5
Total	100.0	100.0	100.0	100.0	100.0

* RM with simultaneous mineral replacement
** Vitamin mix: Vitamin A, 1 200 000 IU/kg; Vitamin D_3, 200 000 IU/kg; Vitamin E, 20 000 IU/kg; Vitamin B_1, 8 000 mg/kg; Vitamin B_2, 8000 mg/kg; Vitamin B_6, 5000 mg/kg; Vitamin B_{12} 1%, 2000 mcg/kg; Niacin, 40000 mg/kg; Calcium Pantothenate, 20000 mg/kg; Biotin, 40 mg/kg; Folic Acid, 1800 mg/kg; Ethoxyquin, 500 mg/kg

The growth trial was conducted using a recirculating system employing biological and mechanical filtration (pebbles and fiber fill). Water was recirculated from the chamber to 50 L capacity aquaria at a flow rate of approximately 600 mL/min/tank. Water quality parameters for the duration of the experiment were: salinity (25-27 ppt), temperature (25–27 °C), pH (8.5-9.0), and dissolved oxygen (>5 ppm); they were monitored weekly. Nitrite (0.000-0.015 ppm) and total ammonia-nitrogen (TAN) (0.00-0.02) were measured using commercially available kits (CP Aqua Test kits). Every 5-7 days, 100% of the water in the chamber was changed. Each aquarium was provided with adequate aeration and cleaned daily by siphoning uneaten feed and feces before feeding.

Twenty five post larvae shrimp (average body weight= 0.03±0.00 g) were distributed randomly into 20 substrate-free 50 L culture tanks. The shrimp were further acclimated to the experimental conditions for 5 days while fed with the control diet. The experimental diets were fed to 4 replicates in a completely randomized design. Feeding was three times daily (08:00, 12:00 and 16:00) for 60 days at a ratio starting at 20% of average wet body weight and decreasing to 6% towards the end of the experimental period. Sampling was conducted on Day 0 and every 15 days thereafter; shrimp from each tank were counted and bulk-weighed.

Estimation of growth and feed efficiency. The parameters measured were weight gain (wg), specific growth rate (SGR), protein efficiency ratio (PER), per cent survival, feed conversion efficiency (FCE), and protein gained (PG). These were calculated using the following formulae:

$$WG(g) = FBW - IBW$$

$$SGR\ (\%/day) = 100*(\ln FBW - \ln IBW)/D$$

$$\%\ FCE = 100*(FBW-IBW)/FI$$

$$PG\ (g) = (CP_f \times FBW) - (CP_i \times IBW) \times 100$$

$$PER = (FBW-IBW)/(FI \times FP)$$

$$Survival,\ \% = 100*Final\ count\ of\ shrimps/initial\ count\ of\ shrimps$$

Where: FBW = final body weight (g) of individual fish
D = days of culture
IBW = initial body weight (g) of individual fish
FP= Feed Protein (in decimal)
CP_i= Initial carcass protein (in decimal)
CP_f= Final carcass protein (in decimal)
FI= total feed intake of individual fish for the whole duration of the experiment

Analytical Methods and Calculations. Samples of the diets were submitted to Oversea Laboratory at San Fernando, Cebu City, Philippines for proximate analysis, while at the University, shrimp body composition analysis were conducted for dry matter, crude protein, and crude fat analysis following standard protocol (AOAC, 1990). Dry matter was determined by placing the sample in a constant weighed crucible and dried in an oven for about 6-24 h at 100°C. Crude fat was determined using Foss®Soxtec 2055. Ash content was determined after incineration in a muffle furnace at 550°C for 12 h (AOAC, 1990). Crude protein was measured after block digestion and steam distillation using Kjeltec digestion system set at a temperature of 400°C and Foss KjeltecTM 8200 auto-distillation unit.

Statistical Analysis. Data were analyzed using Statistical Analysis Software Program (SPSS) version 16. Data were presented as mean ± standard error of the mean (SEM) for each dietary treatment. The data were analyzed by the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. Data that passed these tests were subjected to one way Analysis of Variance (ANOVA); those that did not were transformed until they did and subsequently subjected to one-way ANOVA test. Tuckey-Kramer Honest Significant Difference test for mean separation was used to evaluate significant differences ($p< 0.05$) among treatment means (Lentner and Bishop, 1993). In comparing shrimp fed with or without simultaneous mineral replacement, Student t-test was used at $\alpha=0.10$. The relationship between experimental diets and the shrimp carcass composition were analyzed statistically using Pearson's correlation coefficient (Le, 2003).

Results

The proximate composition of *R. riparium* is shown in Table 2. Crude protein was at 15.55%. Crude lipid, crude fiber, and nitrogen free extract (NFE; digestible carbohydrates) were approximately recorded at 0.21%, 20% and 28%, respectively. Ash content (35.72%) was noted to be half of total dry matter.

Table 2. Proximate composition of *Rhizoclonium riparium var. implexum* Meal (RM).

Composition	
Moisture	7.43
Crude Protein	15.55
Crude Lipid	0.21
Crude Fiber	19.77
Ash	35.72
Nitrogen-free Extract (NFE)	28.75

All values are presented on a dry matter basis. Values are expressed as mean ± SEM.

Survival rates observed were excellent (93-98%) and were statistically similar for all treatments ($p>0.05$). FABW values of shrimp fed diets containing 5.25% and 10.50% were significantly different from each other, with the former being higher than the latter; values of the two other shrimp groups fed the other diets were statistically similar for both of these dietary treatments (Table 2). The same pattern of significance was also observed for SGR and PG values. FCE values were also highest in shrimp fed the diet containing 5.25% RM while the PER values were all higher in those fed the diets containing RM than in those fed the control diet.

Table 2. Growth performance of *Penaeus vannamei* fed experimental diets containing increasing inclusion levels *of Rhizoclonium* raw meal.
FABW = Final Ave. Body Weight; WG= Weight Gain; FCE= Feed Efficiency Conversion;

Treatment	Parameters						
	FABW	*WG*	*SGR*	*FCE*	*PER*	*PG*	*%SURV*
Control	0.86 ± 0.08[ab]	0.85 ± 0.03[a]	5.6 ± 0.07[ab]	0.55 ± 1.4[ab]	1.4 ± 0.05[a]	0.53 ± 0.02[ab]	98.0 ± 2.0[a]
5.25% RM	1.04 ± 0.07[b]	0.98 ± 0.08[a]	5.9 ± 0.11[b]	0.56 ± 1.1[b]	1.5 ± 0.03[ab]	0.62 ± 0.02[b]	97.0 ± 1.0[a]
10.50% RM	0.82 ± 0.07[a]	0.80 ± 0.07[a]	5.5 ± 0.14[a]	0.52 ± 0.87[ab]	1.6 ± 0.03[b]	0.48 ± 0.04[a]	98.0 ± 1.2[a]
15.75% RM	0.92 ± 0.03[ab]	0.88 ± 0.03[a]	5.7 ± 0.05[ab]	0.55 ± 0.61[ab]	1.6 ±0.03[b]	0.54 ± 0.04[ab]	96.0 ± 1.6[a]

SGR= Specific Growth Rate; PG= Protein Gained (g); PER= Protein Efficiency Ratio; Surv= Survival. Values are expressed as mean ± SEM. Mean values with the same lower-case letter are insignificantly different (P> 0.05).

Replacing dietary mineral component with RM did not significantly affect growth and feed efficiency as well as the survival rate of the shrimp post larvae (*p*>0.05) as shown in Table 3. No clinical deficiency was observed in shrimp fed 15.75% RM inclusion level with simultaneous mineral replacement.

Table 3. Growth and feed efficiency of *Penaeus vannamei* fed the experimental diets containing 15.75% RM with or without simultaneous mineral replacement.

Treatment	Parameters						
	FABW	*WG*	*SGR*	*FCE*	*PER*	*PG*	*SURV*
15.75% RM	0.92 ±0.03[ab]	0.88 ± 0.03[a]	5.7 ± 0.05[ab]	0.55± 0.61[ab]	1.6 ±0.03[b]	0.50±0.04[ab]	96.0 ± 1.6[a]
15.75% RM*	0.85 ±0.02[ab]	0.83 ± 0.03[a]	5.6 ± 0.04[ab]	0.51 ± 1.1[a]	1.5 ± 0.03[ab]	0.49 ±0.01[a]	93.0 ± 1.9[a]

* RM with simultaneous mineral replacement. Values are expressed as mean ± SEM. Mean values with the same lower-case letter are insignificantly different (P> 0.05).

Post-larval shrimp groups did not vary significantly in body composition with the dietary treatments (Table 4). Despite this, there were patterns in body composition of *P. vannamei* relative to the diet. There was a highly significant negative correlation between dietary moisture and body moisture (*r*=-0.816), and dietary ash and body moisture (*r*=-0.583).

Table 4. Proximate composition of *Penaeus vannamei* post larvae fed graded levels of *Rhizoclonium* raw meal or simultaneous mineral replacement.

	Moisture	*Crude Protein*	*Crude Lipid*	*Ash*
Initial carcass	17.3 ± 0.30	56.6 ± 2.2	4.14 ± 0.0	3.9 ± 0.04
		Final carcass		
Control	20.7 ± 0.59[a]	63.7 ± 2.7[a]	5.6 ± 2.2[a]	4.0 ± 0.10[a]
5.25% RM	23.4 ± 0.37[b]	62.1 ± 1.9[a]	7.3 ± 1.2[a]	3.9 ± 0.22[a]
10.50% RM	22.3 ± 0.42[ab]	60.4 ± 3.1[a]	4.5 ± 2.3[a]	4.0 ± 0.08[a]
15.75% RM	22.8 ± 0.38[b]	60.4 ± 3.8[a]	8.4 ± 1.9[a]	4.3 ± 0.12[a]
*15.75% RM**	23.3 ± 0.15[b]	60.2 ± 1.8[a]	5.8 ± 0.5[a]	4.0 ± 0.09[a]

* RM with simultaneous mineral replacement. Values are expressed as mean ± SEM. Mean values with the same lower-case letter are insignificantly different (P> 0.05). Data presented as percent dry basis

Table 5. Correlation matrix of whole body composition and proximate composition of the diets (α=0.1) after the 60-day feeding trial.

	Correlation coefficient (r)			
	Body Composition			
Diet	*Moisture*	*Crude Protein*	*Crude Fat*	*Ash*
Moisture	-0.816***	0.435	0.309	0.056
Crude Protein	0.357	-0.011	-0.088	-0.292
Crude Fat	-0.111	0.252	0.095	-0.027
Ash	-0.583**	0.138	0.284	0.281

Whole body composition expressed in g/kg fresh weight. * is 0.1>p>0.05 (marginally significant); ** is 0.01>p>0.05 (significant)
*** is 0.01>p>0.00 (highly significant) (Le, 2003)

Discussion

Despite the low protein content of *Rhizoclonium riparium var. implexum* raw meal and considerable fiber and ash content, it could be a candidate for incorporation into aquafeeds to replace a considerable proportion of soybean meal. In previous studies, we evaluated *Rhizoclonium riparium var. implexum* raw meal for its biophysical properties and digestibility in the diet of Pacific white shrimp, *P. vannamei* (Bunda et al 2015; Bunda, 2015). Seaweed meal is digestible for shrimp with an estimated apparent digestibility coefficient (ADC) of 59.79% for dry matter, and contains a balanced amount of EAAs (essential amino acid index, EAAI, of 0.97 (Bunda et al., 2015b). Hence, this seaweed is a good candidate for a complementary protein source in the diet of white shrimp.

The feeding trial in the present study demonstrated that a wide range of replacement levels (15%-45% soybean meal replacement) could equal or surpass growth and feed utilization performance of shrimp which were fed the control diet. One possible explanation could be that Ulvoids, which include *Ulva* and related genera *Rhizoclonium,* contain xylans and sulphated galactans (Makkar et al. 2016) which are either immunomodulatory, or growth promoting, or both in shrimp.

PER is a parameter that compares experimental diets on an equal unit of protein since it is a measure of weight gain/unit protein fed. PER values of all RM-incorporated diet groups were significantly higher than the control group despite its higher dietary protein content. This indicated that the slight to moderate variation in protein content of the diets did not affect growth probably because of the compensatory increase in voluntary feed intake. This may be due to the appeal of the RM ingredient. Another factor could be that *Rhizoclonium* meal is a good complementary protein source since it exhibits an EAAI of 0.97 implying a balanced diet, despite being deficient in phenylalanine and methionine (Bunda et al., 2015).

The observation that RM meal could wholly replace dietary minerals without deleterious effects on growth, feed efficiency, body composition, as well as survival of shrimp could be attributable to the high ash content of *Rhizoclonium* meal (35.72%). A similar finding was reported by Naorbe et al (2015) when *C. calcitrans* paste was used to simultaneously replace 30% protein of squid meal, 55% vitamins, and 100% minerals in the diet of *P. monodon* post-larvae. Marine macroalgae concentrate minerals from seawater and contain 10-20 times the minerals of land plants thus, making them significant sources of valuable minerals for nutrition (Makkar et al 2016; Mišurcová 2012; Moreda-Piñeiro et al 2012).

Patterns in body composition of the experimental shrimp, as indicated by their correlation matrix in Table 5, revealed that higher dietary ash contributed by dietary RM influenced the composition of other nutrient categories of the shrimp body expressed as dry weight; this could be by way of influencing body moisture.

In conclusion, the present study demonstrated that *Rhizoclonium riparium var. implexum* meal could be a complementary protein source and could replace from 15% to 45% of imported soybean meal (SBM) in the diet of *P. vannamei* post-larvae. At the maximum inclusion of 15.75% RM meal, most growth and utilization parameters were statistically similar or better in those groups fed diets containing RM than the group fed the control diet. Furthermore, an additional 1% over the maximum inclusion level is recommended since there was no deleterious effect on the performance of the shrimp.

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