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Optimum dietary inclusion of *Ulva intestinalis* to the diet of the black tiger shrimp *Penaeus monodon* postlarvae

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Abstract. A study was conducted to evaluate the effects of incorporating *Ulva intestinalis* meal into the feed for the black tiger shrimp *Penaeus monodon* larvae for four weeks. Five diets were prepared, namely diets with no seaweed inclusion (0%, control diet), 5.25%, 10.5%, 15.75% and 21.0% with equivalent replacement rates of soybean meal at 0, 15%, 30%, 45% and 60%. Feeding the experimental diets to postlarval shrimps resulted in no significant differences in survival rate, and food conversion ratio (FCR). However, both weight gain (WG) and specific growth rate (SGR) were highest in shrimps fed the diet containing 15.75% while feed intake (FI) was highest in 10.5%. Estimated optimum level of inclusion was 13.9, 14.1, 14.7% using SGR, FI and WG, respectively; these were equivalent to soybean replacement of 39.7, 40.3 and 42% soybean replacement, respectively. The highest inclusion level of 21.0% did not result in significant difference in the growth performance and feed efficiency of the shrimp juveniles. In conclusion, the optimum inclusion of 13.9 to 14.7% *U. intestinalis* meal was estimated in this study. However, diets containing up to 21.0 % inclusion level could be incorporated in the diet of *P. monodon* postlarvae without deleterious effects on growth and feed efficiency.

Key Words: Soybean replacement, shrimp larvae, green seaweed ingredient, energy supplement.

Introduction. Green algae (division Chlorophyta) are found nearest the shore in shallow waters and usually growing as threadlike filaments, irregular sheets, or branching fronds, containing a number of relatively large forms (*e.g.*, Ulva, Codium, Valonia, Halimeda; Graham 1984). Green algae belonging to the genera *Ulva*, formerly *Enteromorpha* (Hayden et al 2003), are common seaweeds distributed worldwide. Two of the Ulva species have potentials as ingredients for aquafeeds, namely *Ulva* (*Enteromorpha) intestinalis* and *Ulva lactuca.* Both are marine algae although *U. intestinalis* are usually found in brackishwater specifically growing in fishponds. *U. intestinalis* contains lower protein (9.9%), but higher carbohydrates (51%) than that *in U. lactuca* (13.4 and 51.0%, respectively; Santizo et al 2014; Aquino et al 2014). The high carbohydrate in these chlorophytes makes them a good source of energy for aquaculture species. However, biochemical composition of seaweed exhibits variation in nutrient composition which depends on several environmental parameters and ecological conditions such as water temperature, salinity, light, season of the year and the geographic area (Jensen 1993; Dawes 1998; Anantharaman et al 2010; Haroon et al 2000). In addition to being a protein source, chlorophytes have sulphated polysaccharides such as ulvan, which exhibit either immunomodulatory or growth-promoting factor or both, on shrimps *Penaeus monodon* (Declarador et al 2014; Serrano & Declarador 2014; Lauzon & Serrano 2015) and *Penaeus vannamei* (Lauzon & Serrano 2015).

Previously, we have evaluated *U. lactuca* (Chlorophyta) meal and its protein concentrate, for its digestibility and as a replacement for soybean meal in the diet of the black tiger shrimp *P. monodon* (Santizo et al 2014; Serrano & Santizo 2014). The dried raw seaweed and its PC were highly digestible to the shrimp, with an estimated apparent digestibility coefficient of 99.13% and 71.17%, respectively, for dry matter. Evaluation of

U. lactuca in its raw dried form has not been evaluated in *P. monodon* but its PC form has been studied (Serrano & Santizo 2014). Replacing soybean meal with seaweed PC at 15% in the diet resulted in statistically similar survival, food conversion ratio, weight gain and specific growth rate with those fed the control diet (*i.e.* no seaweed content). The green seaweed from brackish water *Ulva* (*Enteromorpha*) *intestinalis* has been evaluated in the Nile tilapia *Oreochromis niloticus* but has not been studied in shrimps. The objective of this study was to evaluate the brackish water green seaweed *U. intestinalis* as a feed ingredient for the diet in the black tiger shrimp *P. monodon*.

Material and Method

Diet preparation. Table 1 shows the ingredient composition and proximate analysis of the 5 experimental diets containing varying amount of *U. intestinalis* meal, namely 0% (control diet), 5.25%, 10.50%, 15.75% and 21.00%. Prior to weighing, all powdered ingredients were made to pass through 150 µm sieve. All dried ingredients were mixed thoroughly before oil and lecithin were added. The bread flour that served as the binder was cooked before it was added to the mixtures. To develop stiff dough, the mixture was thoroughly mixed and passed through a meat grinder twice. The diet was steamed for 15 min to improve the pellet integrity and dried in an oven for 8-12 h at 60°C until the moisture reached <10%. Diets were stored in freezer at -20°C prior to feeding and a portion was subjected to proximate analysis.

Table 1

Composition and proximate analysis of experimental diets containing raw dried *Ulva* (*Enteromorpha) intestinalis* for *Penaeus monodon* growth trial (g 1000 g diet^{-1})

Ingredients	*Control (0%)*	*Diet 1 (5.25%)*	*Diet 2 (10.5%)*	*Diet 3 (15.75%)*	*Diet 4 (21.0%)*
Danish FM	300.0	300.0	300.0	300.0	300.0
Squid meal	29.0	29.0	29.0	29.0	29
CMC	80.5	80.5	80.5	80.5	80.5
Lignobond	15.0	15.0	15.0	15.0	15
Vitamin mix[1]	10.0	10.0	10.0	10.0	10
Mineral mix[2]	10.0	10.0	10.0	10.0	10
DicalPhos	20.0	20.0	20.0	20.0	20
BHT	0.5	0.5	0.5	0.5	0.5
Bread flour	11.7	11.7	11.7	11.7	11.7
Cod liver oil	63.0	63.0	63.0	63.0	63
Lecithin	5.0	5.0	5.0	5.0	5
Soybean meal	350.0	297.5	245.0	192.5	142.5
U. intestinalis meal	0.0	52.5	105.0	157.5	210
Total	1000.0	1000.0	1000.0	1000.0	1000
Estimated gross energy (cal g^{-1})[3]	4021.0	3890.8	3796.5	3753.5	3739.2
Proximate analysis (%, dry weight basis)					
Moisture	10.4	8.4	8.1	7.9	7.6
Crude protein	45.8	43.3	40.59	38.1	35.7
Crude fat	11.6	10.9	10.1	10.1	10.7
Crude fiber	2.8	2.6	2.6	2.6	2.6
NFE	27.9	29.4	33.0	33.0	33.6
Ash	11.0	12.6	13.9	14.9	16.0

[1]Vitamin mix (mg kg^{-1} dry diet unless otherwise stated): Vitamin A 1 200,000 IU, Vitamin D3 200,000 IU, Vitamin E 20,000 IU, Vitamin B1 8,000 IU, Vitamin B2 8,000 IU, Vitamin B6 5,000 IU, Vitamin B12 2000 µg, Niacin 40,000 µg, Calcium pantothenate 20,000 µg, Biotin 40 µg, Folic acid 1,800 µg, Ethoxyquin 500 µg, Carrier q.s ad to make 1 kg; [2]Mineral mix (mg kg^{-1} dry diet unless otherwise stated): Iron 400 mg; Manganese 100 mg; Zinc 400 mg; Copper 40 mg; Iodine 18 mg; Cobalt 0,2 mg; Selenium 2 mg; [3]Estimated based on the physiological fuel of 5.64, 9.44, and 4.11 kcal g^{-1} for protein, lipid, and carbohydrate, respectively (NRC 1993).

Experimental system and feeding trial. *P. monodon* (poslarva 12 or PL 20 *i.e.* 12 days after hatching) was purchased from a commercial hatchery located at Tigbauan, Iloilo. Prior to the purchase, samples were taken from the commercial hatchery and tested for the presence of the white spot syndrome virus (WSSV) by nested polymerase chain reaction (PCR); the pathogen was not detected in the samples. The shrimp postlarvae were transported to the Aquaculture Multispecies Hatchery of the University of the Philippines Visayas in Miagao, Iloilo which was approximately 20 km away. Upon arrival, the shrimp postlarvae were shortly acclimatized by letting the oxygenized polyethylene bags containing the shrimps float on the water of the tank where the shrimps were to be stocked. When the temperature of the water in the two media became similar, the shrimps were very slowly released in the 10 tonner holding tank. The shrimps were maintained in the holding tank until they reached PL 20 (initial average body weight of 0.013 g), sorted out and acclimatized to their experimental containers for 10 days.

The feeding trials were conducted at the Institute of Aquaculture Multispecies Hatchery in a static system for 4 weeks. This shorter culture period than the most commonly practiced 8-12 weeks period was within the suggested culture period by Lazo & Davis (2000) for larvae, such as that in the present study. Lazo & Davis (2000) recommend 14-28 days for larvae (or postlarvae in shrimps), 6-8 weeks for juveniles and 14-18 weeks for larger fish.

The experiment was conducted in 45 x 30 x 37 cm glass aquaria in a completely randomized design, with 5 dietary treatments each with 3 replicates. Each container was filled with 30 L of water (salinity of 18-20 ppt) in the static system which was changed every 2 days. PL 20 shrimps (initial average body weight, ABW, of 0.013 g) were stocked into the aquarium at a density of 1 individual per liter (*i.e.* 15 individuals tank^{-1}). Shrimps were fed initially at a rate of 20% of ABW divided into 6 equal portions until the termination on the 4th week. Uneaten feeds were estimated before siphoning the water off. Fecal matter and uneaten feed were siphoned off before feeding in the morning. Total ammonia-N and nitrite of the water were monitored to have a range of 0.1-0.2 and 0.3-0.6 parts per million (ppm) respectively; the values were within the optimal conditions for the shrimp postlarvae. Counting and bulk-weighing was done every 15 days and the feeding rate for the next two weeks was adjusted. Culture tanks were cleaned daily and 30-50% of total volume of water from the chamber of the recirculating system was replaced. At the end of the culture period, shrimps in every treatment were weighed, pooled, sacrificed and subjected to carcass proximate analysis.

Response parameters. Growth performance and feed utilization were evaluated: weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate. These parameters were estimated using the following formula:

$$\text{SGR (\% daily)} = (\ln \text{FBW} - \ln \text{IBW}) / \text{D} \times 100$$

Where: FBW - final body weight, IBW - initial body weight, D - number of days of culture.

$$\text{Weight gain (g)} = \text{IBW} - \text{FBW}$$

$$\text{Food conversion ratio (FCR)} = \text{feed consumed (g)/wet weight gain (g)}$$

$$\text{PER} = \text{weight gain (g)/protein fed (g)}$$

$$\text{Survival rate} = 100 \times (\text{final number of shrimp / initial number of shrimp})$$

Chemical, mathematical and statiscal analysis. Seaweed ingredients and experimental diets as well as initial and final carcasses were analyzed for proximate composition. Moisture was measured using a thermo-balance (Mettler Toledo HB43 halogen moisture analyzer). Ash content was determined after incineration in a muffle furnace at 550°C for 12 h (AOAC 1995). Crude protein was measured after block digestion and steam distillation using Foss Tecator™ digestion system and Foss Kjeltec™ 8200 auto-distillation unit. Crude fat was extracted using Foss Soxtec™ 2050 automatic system and fiber was determined using Foss Fibertec™ 2010 system.

Data were analyzed by using a quadratic model into which the response data obtained were fitted to obtain the optimum dietary inclusion of *U. intestinalis*; the model was used to estimate protein and amino acids (Chiu et al 1988; Zeitoun et al 1976). This model was deemed appropriate for the treatment of almost a hyperbolic data outside the control treatment in which the response parameters reached a peak and declined to exhibit a possible toxic effect. The following generalized quadratic equation was used:

$$R = a + bI + cI^2$$

Where: R - measured response, I - dietary nutrient concentration, and a, b, and c - constants that are calculated to provide the best fit of the data. The value of I that produces the maximum response I_{max} is calculated as follows:

$$I_{max} = -0.5\ (b\ /c)$$

Statistical analysis*.* Statistical analysis was performed using Statistical Analysis Software Program (SPSS) version 16. Data were presented as standard deviation (SD) for each dietary treatment. To classify whether the method that should be used was parametric or non-parametric, data were subjected to Kolmogorov-Smirnov test for normal distribution and Levene's test for homogeneity of variances. If the data passed these tests, a one-way analysis of variance (ANOVA) test was conducted on the data on survival, final ABW, SGR and FCR. If the data did not pass either tests, they were transformed until they passed both tests. When ANOVA results showed significant differences, Tukey's Test was performed to rank the means of the responses at $\alpha=0.05$.

Results and Discussion. Proximate analysis of each experimental diet fed to *P. monodon* postlarvae is shown in Table 1. Since the *U. intestinalis* meal (crude protein or CP = 9.9%, Aquino et al 2014) replaced partially the soybean meal (CP ~40%), there were differences in the protein content of the experimental diets. Despite the differences in the CP of the experimental diets, growth increased as the inclusion level increased until 15.75%, beyond which growth decreased albeit non-significantly. Growth (*i.e.* WG and SGR) and feed efficiency (*i.e.* FCR and PER) were generally similar in shrimps fed the control and test diets despite the decreasing CP as seaweed inclusion level increased. This observation was tied with feed intake (FI) which exhibited a second-degree polynomial relationship with the inclusion level *i.e.* feed intake increased and exhibited a peak near the computed optimum requirement. Changes in FI could be an indicator of attractability of the macroalgae at a certain inclusion level. The lowest inclusion level of 5.25% probably was not sufficient to elicit attractability of the diet while the highest inclusion of 21.0% contained excessive ash and carbohydrates that decreased the nutritive value of the diet and perhaps affected its attractability.

The present study confirmed that growth performance of *P. monodon* was either not affected or improved when *U. intestinalis* meal was incorporated in their diet. For the range of inclusion of the seaweed (5.25 to 21.0%), no clinical deficiency was observed; SGR was generally higher in shrimps fed with the test diets than those fed with the control diet. As shown in Table 2, FI, WG, FCR and PER of shrimps fed the experimental diets were statistically similar with those fed the control diet. The results in the present study differed from the previous results using *U. lactuca* meal as a replacement for soybean meal in *P. monodon*. Inclusion of 5.2 and 10.2% (equivalent to 15 and 30% soybean replacement, respectively) did not statistically reduce final ABW, feed intake, feed conversion efficiency and protein efficiency ratio (Serrano et al 2015). However, SGR and protein gained in the previous study were significantly lower in shrimps fed 10.5% seaweed than in those fed the control diet.

Table 2

Growth performance of the shrimp *Penaeus monodon* fed diets containing various levels of *Ulva intestinalis* meal

Parameter	*Control (0%)*	*Diet 1 (5.25%)*	*Diet 2 (10.5%)*	*Diet 3 (15.75%)*	*Diet 4 (21.0%)*
IBW	0.01	0.01	0.01	0.01	0.01
FBW	0.14 ± 0.03^{ab}	0.11 ± 0.00^{a}	0.14 ± 0.00^{ab}	0.16 ± 0.02^{b}	0.12 ± 0.11^{ab}
FI	0.11±0.01	0.10±0.01	0.14±0.01	0.12±0.01	0.10±0.00
WG	0.08±0.01	0.07±0.00	0.09±0.01	0.09±0.01	0.08±0.00
SGR	4.78 ± 0.21^{ab}	4.48 ± 0.07^{b}	5.09 ± 0.09^{ab}	5.19 ± 0.16^{a}	4.80 ± 0.14^{ab}
Surv.	84.4±8.0	75.6±8.9	84.5±9.7	77.8±8.0	91.1±2.2
FCR	1.46±0.31	1.47±0.23	1.54±0.10	1.24±0.03	1.35±0.05
PER	0.04±0.01	0.04±0.01	0.05±0.00	0.04±0.00	0.03±0.00

IBW - initial body weight (g), FBW - final body weight (g), FI - feed intake fish^{-1} (g), WG - weight gain (g), SGR - specific growth rate (% body weight day^{-1} fish^{-1}), Surv. - survival rate (%), FCR - food conversion ratio, PER - protein efficiency ratio. Values are means of triplicate groups, values in the same row not sharing a common superscript are significantly different ($p<0.05$).

PER is a parameter that compares the experimental diets on an equal unit of protein; the statistically similar PER of shrimps in all treatments showed that the differential protein content did not affect growth perhaps because of the compensatory increased in feed intake. In addition, the protein of *U. intestinalis* could be similar with that of another chlorophytes such as *Rhizoclonium riparium* var implexum which exhibited essential amino acid index (EAAI) for the shrimp *P. monodon* of 0.97 (Bunda et al 2015), an indication of well-balanced essential amino acids which probably influenced its attractability. Essential amino acids of *Ulva* proteins accounted for 30.8-33.6% of total amino acid content (Shuuluka et al 2013) which was comparable to those of eggs, soybean, other *Ulva* species and green seaweed proteins: 36.1% in ovalbumin, 37.0-37.9% in *Ulva pertusa* and *Codium fragile* (Fujiwara-Arasaki et al 1984), 37.1% in *U. lactuca* (Ochiai et al 1987), 36.5-38.6% in *Ulva rigida* and *Ulva rotundata* (Fleurence et al 1999), 31.1-48.3% in *Rhzoclonium riparium* var implexum (Bunda et al 2015), 33.6% in leguminous plants (Fowden 1954), and 40-42% *Ulva clathrata* (Pena-Rodriguez et al 2011).

A more refined estimate was provided by quadratic equation regression which provided the estimate of the optimum dietary inclusion of 13.9%, 14.7% and 14.1% using SGR, WG and FI, respectively) or an equivalent of 39.7%, 42.0% and 40.3%, replacement of soybean meal with the seaweed, respectively. Fitting the responses into the quadratic model gave regression equations of $y = -0.0097x^2 + 0.270x + 2.7425$, $r = 0.979$ for SGR, $y = -0.0006x^2 + 0.0176x + 0.035$, r = 0.917 for WG and $y = -0.0009x^2 + 0.0253x + 0.0812$, $r = 0.96$ for FI.

As estimated by fitting the data into a polynomial curve (Figure 1), soybean meal could be replaced by *U. intestinalis* in the range of 39.7%-42.0%. The growth performance of the group fed with 21.0% dietary inclusion did not differ significantly with the control group (0% inclusion) (Table 2). Furthermore, survival was not affected by the levels of *U. intestinalis* inclusion. Few studies have dealt with incorporating macroalgae in shrimp diets and they differed in optimum levels of inclusion. Peñaflorida & Golez (1996) observed good weight gain when shrimp postlarvae (ABW = 0.2 g) diets containing 5% *Kappaphycus alvarezii* and 3% *Gracilaria heteroclada*; feeding larger shrimps (500 mg) did not have any effect on growth performance. Rodríguez-González et al (2014) fed the white leg shrimp *Litopenaeus vannamei* postlarvae with diets containing *U. lactuca* or *Gracilaria parvispora* meals. Diets containing 10 and 15% *U. lactuca* resulted in significantly lower growth than did shrimps fed diets without inclusion. However, shrimps fed with diets containing *G. parvispora* up to 15% inclusion did not differ from those fed the control diet (without inclusion). Inclusion of 20% *Microcystis* meal resulted in negative growth of juvenile *L. vannamei* (Rivera et al 2002). Feeding *P. monodon* with diets containing *Gracilaria* spp. resulted in reduced growth. Feeding *U. lactuca* meal to *P. monodon* postlarvae at 10.5% inclusion level (equivalent to 30% soybean replacement) could be used in the diet without deleterious effects (Serrano et al 2015). In the present study, feeding diets containing the highest inclusion of 21.0% did not significantly differ in the SGR, final ABW and FCR of *P. monodon* juvenile. This was despite the gradual lowering of protein contents of the diets as the inclusion level of the seaweed meal increased. This suggests that a higher than the optimum level (14.7%) of inclusion of these green seaweed could be used without deleterious effect on growth and feed efficiency of the shrimp.

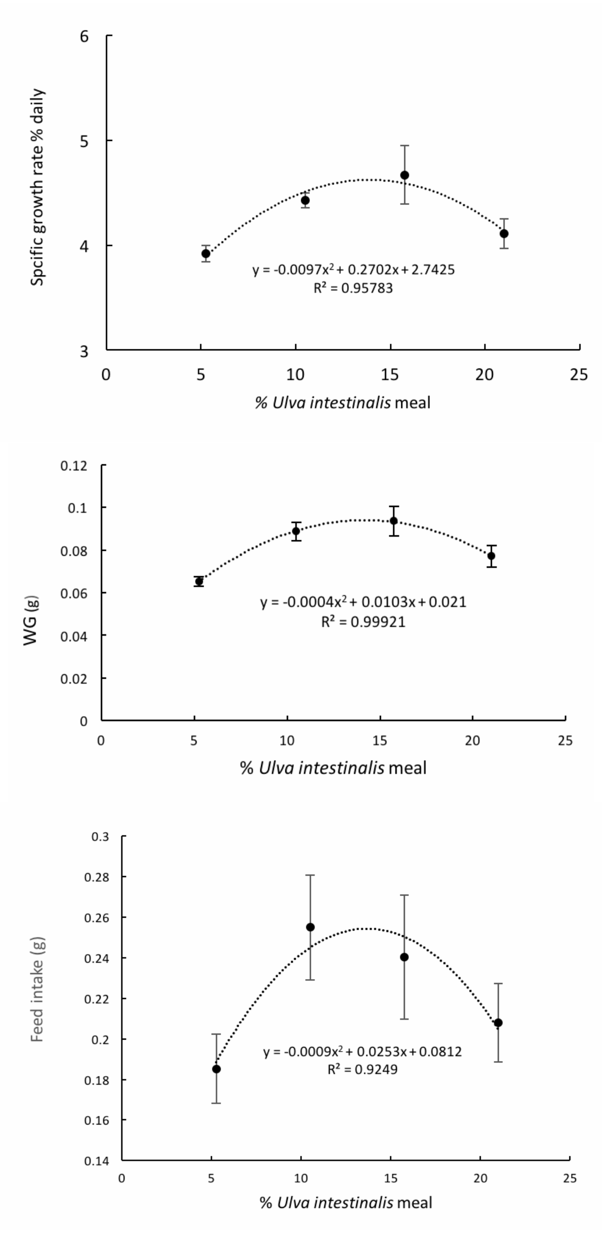

Figure 1. Responses of the shrimp to varying dietary *Ulva intestinalis* inclusion fitted into quadratic model. The uppermost figure above is the specific growth rate (SGR) response, the middle is the weight gain (WG) response and the lowermost is the to varying dietary seaweed inclusion. Optimum dietary inclusion (I_{max}) estimated using SGR was 13.9%, WG was 14.7%, and feed intake was 14.1% which were equivalent to 39.7%, 42.0% and 40.3% replacement of soybean meal with the *U. intestinalis* meal, respectively.

Conclusions. The present study was able to estimate the optimum inclusion level of 13.9 to 14.7% or equivalent to 39.7% to 42% replacement of soybean meal in the diet of *P. monodon* postlarvae. However, the highest inclusion of 21.0% or 60% replacement of soybean meal in the diet could be used without deleterious effects on the growth performance and feed efficiency of the shrimp.

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