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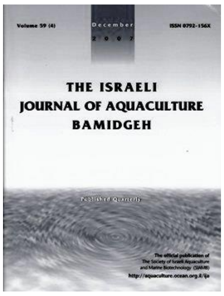

Nutritional Evaluation of *Rhizoclonium riparium* var *implexum* Meal to Replace Soybean in the Diet of Nile Tilapia Fry

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Keywords: crinkle grass; seaweed meal; substitute ingredient; sex-reversed tilapia

Abstract

A study was conducted to evaluate the potential of crinkle grass *Rhizoclonium riparium var implexum* meal (RM) as a substitute for soybean in the diet of sex-reversed Nile tilapia *Oreochromis niloticus* fry. Four experimental diets were prepared containing various inclusion rates of RM at 0% (Diet 1), 4.2% (Diet 2), 8.4% RM (Diet 3) and 12.6% (Diet 4) equivalent to 0%, 15%, 30% and 45% replacement of soybean meal, respectively. Results showed that Diet 4 resulted in significantly higher final average body weight (FABW), weight gain (WG), protein efficiency ratio (PER), and better feed conversion ratio (FCR) than Diet 1. Diets 2 and 3 resulted in statistically similar responses to those of both Diets 1 and 4. Specific growth rate (SGR) was not affected by the dietary treatments. Dietary proximate compositions were correlated with the performance indices. This study showed that *Rhizoclonium riparium* var. *implexum* meal could be included in the diet of Nile tilapia up to 12.6%, equivalent to 45% soybean meal replacement. This could be an important cost-effective measure for intensive culture of tilapia.

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Introduction

The aquaculture industry has grown rapidly in the last decades, contributing a steady increase to the gross national product of many world economies. Almost half of all fish for human consumption has been provided by the industry and this is predicted to grow as demand for, and the supply of aquatic products increase and natural sources decrease (FAO, 2014).

The farming of cichlids especially tilapia is the most widespread branch of aquaculture in the world (FAO, 2014). In the Philippines, tilapia is one of the most commonly cultured species, along with milkfish and shrimp. It is highly favored by aquaculturists as it can tolerate wide ranging environmental conditions, grows rapidly, reproduces effectively, and feeds at different trophic levels.

Aquaculture production is limited by the cost of feeds specifically because some ingredients are imported. Soybean meal is the major ingredient in formulated diets, and could replace a large portion of fishmeal as a source of protein, however it is subject to price fluctuation. Soybean meal is also necessary in the formulation of farm animal feeds thus competing for supply and affecting price rises. Consequently, cost of fish production and long-term sustainability are affected. Fish nutritionists are striving to find alternative plant protein sources such as lupin, pea, and cowpea meals, which would replace soybean which in turn is used to partially or wholly replace fishmeal in aquafeeds. The production of soybean meal in the Philippines is barely sufficient for human consumption therefore importation is necessary. As a consequence, soybean prices fluctuate, fish production may be reduced, leading to lack of food security.

Algae are candidates for complete or partial substitution of soybean meal in aquafeeds. They can be fed fresh or processed as meal, mixed in the diet of finfish, crustaceans, and mollusks. Seaweeds are beneficial due to their nutritive value, high rate of production, and immunostimulatory effects (Declarador et al., 2014). Most common seaweeds are not utilized and are considered contaminants in water recreation areas. *Rhizoclonium sp.*, a filamentous algae belonging to the Cladophoracea family, is a potential aquafeed ingredient. It was successfully used as a feed supplement in a composite algal mix fed to *Oreochromis mossambicus* (Roy et al. 2011). Although there are indications that *Rhizoclonium riparium var implexum* can be mass produced (Chao et al., 2004), no data is available regarding its utilization as a feed ingredient for the Nile tilapia *Oreochromis niloticus*.

Materials and Methods

Preparation of Rhizoclonium meal. Rhizoclonium riparium var implexum was collected from Arevalo, Iloilo City, Iloilo. The algae were transported to the Fish Nutrition Laboratory, University of the Philippines Visayas, Miag-ao, Iloilo and manually cleaned of dirt, snails, and other unwanted matter. The algae were shade-dried, then oven-dried at 60°C for 24 h, pulverized using a mechanical grinder and passed through a 150µ sieve. The powdered *Rhizoclonium* spp. (herein referred to as *Rhizoclonium* meal or RM) was kept frozen at -20°C until use. The experiment was conducted from June to August, 2015.

Diet preparation. Four experimental diets were formulated to contain a range of crude protein from 33%-36%, which fulfilled the requirements of juvenile tilapia. The diets were designed to provide 5%-7% crude fat containing varying amounts of RM. The seaweed meal was included in the diet at various levels: 0% (Diet 1, control), 4.2% (Diet 2), 8.4% (Diet 3) and 12.6% (Diet 4) which were equivalent to soybean replacement levels of 0%, 15%, 30% and 45%, respectively. The basal diet was formulated according to Santiago et al. (1982) (Table 1). The ingredients were pulverized and passed through a 150 µm sieve prior to mixing. All dried ingredients were thoroughly mixed and then the liquid ingredients were added. Gelatinized cornstarch was added before pelletizing. The moistened mixture was pelleted (2 mm) in a meat grinder and oven-dried at 60^{o}C for 16-18 h to about 10% moisture. Diets were then crumbled into appropriate sizes, sealed in plastic bags, and stored at -20^{o}C until use.

Table 1. Feed formulation of experimental diets for *Rhizoclonium* sp. raw meal as partial replacement of soybean meal

Ingredients	*Control*	*4.2%*	*8.4%*	*12.6%*
Fishmeal (65%CP)	200.0	200.0	200.0	200.0
Squid Meal	80.0	80.0	80.0	80.0
Shrimp Meal	80.0	80.0	80.0	80.0
Copra (coconut) meal	100.0	100.0	100.0	100.0
Soybean meal	280.0	238.0	196.0	154.0
Rice bran	120.7	120.7	120.7	120.7
Cornstarch	50.0	50.0	50.0	50.0
Soybean oil	30.0	30.0	30.0	30.0
[a]Vitamin premix	21.7	21.7	21.7	21.7
[b]Trace mineral premix	21.6	21.6	21.6	21.6
CMC	16.0	16.0	16.0	16.0
Rhizoclonium meal	0.0	42.0	84.0	126.0
Total	1000.0	1000.0	1000.0	1000.0
Proximate Analysis (%)				
Moisture	9.2	9.5	7.5	7.0
Crude Protein	36.2	34.0	33.4	33.3
Crude Fat	5.8	5.4	6.9	7.7
Crude Fiber	2.2	5.1	6.3	6.2
NFE	32.5	30.7	28.5	26.0
Ash	14.2	15.2	17.5	19.8
[c]Gross Energy (kcal/g)	3.3	3.1	3.1	3.1

[a] Vitamin mix: Vitamin A, 1,200,000 IU/kg; Vitamin D3, 200,000 IU/kg; Vitamin E, 20, 000 mg/kg; Vitamin B_1, 8000 mg/kg; Vitamin B_2, 8000 mg/kg; Vitamin B_6, 5000 mg/kg; Vitamin B_{12}1%, 2000 mcg/kg; Niacin, 40,000 mg/kg; Calcium Pantothenate, 20, 000 mg/kg; Biotin, 40 mg/kg; Folic Acid, 1,800 mg/kg; Ethoxyquin , 500 mg/kg

[b] Mineral mix: Fe, 40,000 mg/kg; Mn, 10,000 mg/kg; Zn, 40,000 mg/kg; Cu, 4000 mg/kg; I, 1,800 mg/kg; Co, 20 mg/kg; Se, 200 mg/kg

[c] Gross energy estimated according to the following physiological fuel values: 4.0 kcal/g protein, 9.0 kcal/g lipid, and 4.0 kcal/g NFE.

Experimental tilapia and set up. One thousand (1000) sex reversed Nile tilapia (*Oreochromis niloticus*) fry were procured from the Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC-AQD) in Tigbauan, Iloilo, acclimatized to the basal diet and to the laboratory conditions for 10 days. Four hundred Nile tilapia fry were randomly stocked in twenty, 60L tanks (20 fry/tank). The experimental diets were fed to four replicate groups of Nile tilapia fry twice a day. Every 15 days, fish were bulk-weighed, and feeding rate was adjusted for the next 15 days until termination after 8 weeks. The feeding trial was conducted in a closed recirculating system in which approximately 70% of the water in the system was replaced every other day. Uneaten feed and feces were siphoned-off each morning before the first feeding. Chlorinated tap water used for replacement (100 ppm NaClO) was dechlorinated by letting it stand while being aerated for 3 days. Water quality indices were monitored periodically: temperature and pH were measured twice a day, dissolved oxygen (DO) twice a week, and nitrite and total ammonia weekly with commercially available kits.

Growth performance parameters. Growth performance and feed efficiency were calculated using the following formulae (Wilson, 2002):

Weight gain, WG (g) **=** $W_2 - W_1$

Where: W_2 = Final weight (g) of individual fish and W_1 = Initial weight (g) of individual fish

Specific Growth Rate (SGR, %/day) = $[(\ln W_2 - \ln W_1) / (T_2 - T_1)] * 100$

Where T_2 = Final time (in days) T1 = Initial time (in days)

FCE (%) = Total individual FI /weight gain of individual fish

Where Total FI = total feed intake of individual fish for the whole duration of the experiment

PER = weight gain of individual fish / (FI*feed protein (in decimal))

Survival (%) = 100*Final number of fish /container÷ Initial number of fish/ container

Statistical analysis. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 23 software. Data were presented as mean ± standard error of the mean (SEM). The data were analyzed by the Shapiro-Wilk test for normal distribution and by the 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. Growth indices (FABW, WG, SGR), feed efficiency parameters (FCR, PER), survival rates were subjected to one-way ANOVA at α=0.1, and subsequently Tukey's HSD test was performed to rank the mean values. Pearson's correlation coefficients and their p values **(**r_s; p<0.1) were used to determine the relationship between the proximate composition of the diet and growth performance and feed efficiency. In comparing the means and expressing Pearson's correlation coefficient, p values were given and the following levels of significance were used both in the one-way ANOVA and in Pearson's correlation coefficient (Le, 2003): if p>0.10, the result was considered not significant; 0.05<p<0.10, as marginally significant; 0.01<p<0.05 as significant and p<0.01 as highly significant.

Results

The fish readily accepted all experimental diets, indicating acceptable palatability of the *Rhizoclonium* meal. Fish appetites were good and consumption was normal during the entire trial. No feed-related mortalities were observed during the trial.

Proximate composition of the experimental diets. As RM levels increased in the experimental diets there was no significant correlation with crude protein (r=-0.88, p=0.11), gross energy (r=-0.78, p=0.11), crude fat (r=0.89, p=0.11) and crude fiber (r=0.89, p=0.11) (data not shown). In contrast, moisture (r=-0.90, p=0.1), NFE (r=-1.0, p=0.00) and ash content (r=-0.99, p=0.01) showed significant linear correlation with increased levels of RM.

Table 2. Proximate composition of the *Rhizoclonium riparium var implexum* meal.

Proximate composition	*% (dry weight basis)*
Moisture	12.25
Crude protein	13.93
Crude fat	0.56
Crude fiber	17.70
Ash	35.57
NFE	32.23
Gross energy (kcal/kg)	1.90

Growth performance. Growth performance, feed efficiency and survival of sex-reversed *Oreochromis niloticus* fry fed with experimental diets containing increasing inclusion levels of RM is shown in Table 3. Initial average body weight (IABW) was statistically the same (p=0.56) for all dietary treatments. The range of final average body weight (FABW) of tilapia fry was 1.77g-2.64g with no significant differences (p=0.03). Fish fed 12.6% RM diet exhibited the highest FABW (2.64 g) but were not significantly different to those fed Diets 2 and 3 (containing 4.2% and 8.4% RM, respectively). FABW of control diet group was significantly lower than the Diet 4 (12.6% RM) group, but was statistically similar to groups fed Diets 2 and 3.

Table 3. Growth performance and survival of juvenile *Oreochromis niloticus* fed with diets containing increasing replacement levels of soybean meal with *Rhizoclonium* meal (RM)

	%RM	IABW (g)	FABW (g)	WG (g)	FI (g)	SGR(% day^{-1})	FCR	PER	Survival (%)
D1	0	0.05 ± 0.01^a	1.77 ± 0.15^a	1.72 ± 0.15^a	3.27 ± 0.21^a	5.46 ± 0.09^a	1.92 ± 0.06^a	1.31 ± 0.04^a	100.00 ± 0.00a
D2	4.2	0.06 ± 0.01^a	2.17 ± 0.26^{ab}	2.11 ± 0.25^{ab}	3.68 ± 0.25^a	5.39 ± 0.08^a	1.76 ± 0.04^b	1.52 ± 0.04^b	100.00 ± 0.00a
D3	8.4	0.06 ± 0.01^a	2.26 ± 0.14^{ab}	2.0 ± 0.14^{ab}	3.82 ± 0.18^a	5.45 ± 0.08^a	1.75 ± 0.04^{bc}	1.59 ± 0.04^b	100.00 ± 0.00a
D4	12.6	0.06 ± 0.01^a	2.64 ± 0.16^b	2.58 ± 0.15^b	3.85 ± 0.26^a	5.64 ± 0.13^a	1.49 ± 0.03^c	1.88 ± 0.05^c	100.00 ± 0.00a

Values in the same column with different superscript letters are significantly different ($P \leq 0.05$). Values are mean ± SEM. IABW, initial average body weight; FABW, final average body weight; WG, weight gain; FI, total individual feed intake; SGR, specific growth rate; FCR, feed conversion ratio.

Final WG was significantly different (p=0.03) in the dietary groups at the end of the trial. WG in tilapia fry fed Diet 4 (12.6% RM) was highest (3.22 g) but was statistically similar with those fed Diets 2 and 3. Lowest WG was recorded in the control group but it was statistically similar to the Diet 2 and 3 groups. Individual total feed intake (FI) was statistically similar (*p*=0.39) among fish fed the experimental diets with a range of 3.70 g for fish fed the control diet, to 4.36 g for those fed Diet 4. Specific growth rate (SGR) of all fish groups were statistically similar (*p*=0.36).

FCR values between the groups were significantly different (*p*=0.00). Diet 4 (12.6% RM) produced the lowest FCR value while Diet 1 produced the highest FCR value. The fish fed Diets 2 and 3 exhibited intermediate values.

Differences in PER values were highly significant (p=0.00). PER value in the Diet 4 group was significantly highest while PER value in the Diet 1 group was significantly lowest. Diets 2 and 3 were statistically similar and exhibited intermediate PER values. Pearson's correlation analysis (Table 4) concurred well with the performance results in Table 3. Survival rates were 100% in all groups.

Table 4. Matrix of Pearson's correlation coefficients between proximate composition of the diet and the dependent variables (i.e. growth/efficiency performance) of the Nile tilapia after 8 weeks of feeding trial (α=0.1).

Independent variables	*Dependent variables*				
	FABW	*WG*	*SGR*	*FCR*	*PER*
Moisture	-0.82,	-0.81	-0.18	0.83	-0.94*
Crude protein	-0.53,	-0.52,	0.45	0.15	-0.41
Crude fat	0.89	0.89	0.32	-0.88	0.97**
Crude fiber	0.52,	0.51,	-0.46	-0.19	0.44
NFE	-0.43*	-0.44*	-0.21	0.58**	-0.77***
Ash	0.43*	0.44*	0.25	-0.59**	0.77***
Gross energy	-0.38,	-0.37	*r*=0.58	-0.07	-0.20

A Pearson's correlation coefficient gives a value between+1 and−1 inclusive, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation. Significant probabilities are in bold and * is 0.1>p>0.05 (marginally significant); ** is 0.01>p>0.05 (significant) and *** is 0.01>p>0.00 (highly significant) (Le 2003).

Discussion

Chemical composition of RM meal. In the present study crude protein levels of green seaweed RM (13.93%) were similar to results using other green seaweeds (Banerjee et al. 2009; Aguilera-Morales and Perez-Gil 2005) on *Ulva intestinalis* (10.04% dry weight basis) and *Enteromorpha* spp. (10.38% dry weight basis). The CP values in the present study were lower than those in *Enteromorpha compressa* at 13.60% (Wahbeh, 1997) and *Ulva clathrata* 20 to 26% (Peña-Rodríguez et al., 2011).

Nitrogen-free extract (NFE i.e. carbohydrate component) of *Rhizoclonium riparium* var *implexum* in the present study was lower than those reported for *Enteromorpha intestinal* (45.3%, Banerjee et al., 2009) and (51.4%, Benjama and Masniyom 2011). The point of lowest carbohydrate synthesis occurred when protein levels increased and vice versa (Martinez and Rico 2002). Low NFE values in the present study may have been due to the maximal level of CP content of the RM.

Lipid content of seaweed meal *(*0.56%) was lower than for *Enteromorpha intestinalis* at 1%-3% (Mabeau and Fleurence, 1993) and *Enteromorpha linza* at 2.2% (Yildrim et al., 2009). In the present study, the values obtained were higher than those observed in *Ulva* sp. at 0.1% (Ergun et al., 2009) and *Ulva rigida* at 0.2% (Güroy et al., 2007). Lipid content is generally low in seaweeds found in high temperate regions such as the Philippines, as observed in *Enteromorpha intestinalis* (Banerjee et al., 2009). High ash content (35.6%) in the present study was similar to that in *Enteromorpha* sp. at 32%-36% (Aguilera-Morales and Perez-Gil, 2005), *Ulva clathrata* at 38.4% (Cruz-Suarez et al., 2006), *Ulva lactuca* at 32.9% and for *Enteromorpha linza* at 32.6% (Yildrim et al 2009).

Proximate composition of the experimental diets. In general, ingredients which contain 20% CP or more are considered protein sources while those below are considered energy sources. Since the CP content of *Rhizoclonium riparium* contained less, it is considered an energy source unless processed as a concentrate.

Body carcass analysis was not carried out in the present study due to insufficient dried body samples at the termination of the experiment. Nonetheless, it was observed that dietary composition influenced certain performance indices of the fish (Table 4). The negative linear relationship between dietary moisture (thus, proportional relationship with dry matter, DM) with PER suggested that the highest DM intake (Diet 4) resulted in maximal weight gain per unit of protein intake. There was no significant difference in performance indicators in Nile tilapia juveniles although dietary protein was highest in the control diet. Enhancement of 2.2-2.9% crude protein does not appear to affect response to *Rhizoclonium riparium* (Table 3) and RM does not appear to promote growth. Growth promotion was observed in Nile tilapia fry fed diets containing *Enteormorpha intestinalis,* another green seaweed (Aquino et al. 2014). Crude fat varied among the dietary treatments (5.4%-7.7%) and the contribution of the RM was negligible (0.56%) such that even if the PER increased linearly with dietary crude fat, it could not be attributed to the seaweed alone. The lowest NFE content in Diet 4 resulted in the highest FABW, WG, PER and better FCR. Similarly, Nile tilapia fed Diet 1 had significantly lower FABW, WG, FCE (i.e. higher FCR) and PER than fish fed Diet 4.

Feed acceptability. Feeding rate was based on the ABW at the start of the experiment, and every 15th day. Differences in FCR reflect the acceptability of the given feeds by tilapia juveniles. Feed intake increased in Atlantic salmon juvenile fed a diet containing Verdemin, an extract derived from *Ulva ohnoi* (Norambuena et al. 2015). The increase in FCR in fish fed Diet 4 (12.6% RM) may be due to increased FI due to the same extract found in RM.

Growth and feed efficiency performance. FABW was highest in fish fed Diet 4 although the active compound of macroalgae responsible for growth improvement has not been clearly defined. Benefit to growth may be due to vitamin and mineral content, lipid mobilization, and improved absorption and assimilation ratios.

Ulva at low levels (2%-5%) does not inhibit, and may even improve, growth performance and feed efficiency of carnivorous fish as seen in rainbow trout *Oncorhynchus mykiss* (Guroy et al 2011), black sea bream *Acanthopagrus schlegeli* (Nakagawa et al., 1993), Japanese flounder *Paralichthis olivaceous* (Xu et al., 1993) and gilthead sea bream *Sparus aurata* (Emre et al., 2013). Growth performance and feed efficiency improved with addition of the following amounts of *Ulva* meal in diets: up to 15% in red tilapia *Oreochromis* sp. (El-Tawil, 2010), 28% in gray mullet *Liza ramada* fry (Elmorshedy, 2010), 5-15% in common carp *Cyrpinus carpio* (Diler et al., 2007), and 5-10% in Nile tilapia *Oreochromis niloticus* diets (Guroy et al., 2007). In the present study we found that growth and feed efficiency improved with up to 12.6% inclusion of *Rhizoclonium riparium* meal in both herbivorous/omnivorous fish species.

Algae in general have been found to increase absorption and assimilation of dietary protein (Yone et al., 1986) which may explain improved feed efficiency indicators such as FCR and PER in fish fed diets incorporating RM. A previous study was conducted with adult Nile tilapia fed a control diet similar to that in the present study and a test diet containing 30% *Rhizoclonium* meal (Serrano, unpubl). Results were similar to the present study for fish fed the diet containing 30% *Rhizoclonium* meal. There was a significant ten-fold increase in hepatic amylase activity and a seven-fold increase in trypsin-like activity in the pancreas of Nile tilapia fed *Rhizoclonium* meal (RM) compared to the control diet group.

In conclusion, this study showed that *Rhizoclonium riparium* var. *implexum meal* could replace imported soybean meal in Nile tilapia diets. Replacement rate of 12.6%, equivalent to 45% soybean replacement by RM was optimal. Incorporating seaweed such as *Rhizoclonium* spp. in aquafeeds is cost-effective especially in intensive tilapia culture.

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