AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

Protein concentrate of *Ulva intestinalis* (Chlorophyta, Ulvaceae) could replace soybean meal in the diet of *Oreochromis niloticus* fry

[1]Augusto E. Serrano Jr., [2]Jon I. L. Aquino

[1] Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miag-ao, Iloilo, Philippines; [2] Southeast Asian Fisheries Development Council-Aquaculture Department, Tigbauan, Iloilo, Philippines. Corresponding author: A. E. Serrano Jr., serrano.gus@gmail.com

Abstract. An experiment was conducted to evaluate the effects of replacing soybean meal with the protein concentrate of *Ulva intestinalis* (UPC) in the diet of juvenile Nile tilapia, *Oreochromis niloticus*. Four experimental diets were formulated to replace 0, 15, 30 or 45% of soybean meal by weight and were fed to group of fish for 90 days. Results showed that survival rates were high and independent of dietary treatment. Feed intake decreased as level of inclusion of UPC increased. Food conversion ratios of Nile tilapia fry fed the experimental diets were all statistically similar. Weight gain and specific growth rate of Nile tilapia fry fed with the diet containing 15% replacement was statistically similar with those of fish fed with the control diet. Thus, *U. intestinalis* protein concentrate could replace 15% by weight of the imported soybean meal without negatively affecting food conversion ratio, weight gain and specific growth rate; this substitution could mean slightly cheaper formulated diets for the Nile tilapia fry and fingerlings.

Key Words: processed *Ulva*, sea lettuce, Nile tilapia fry diet, cost-effective diet, maximum dietary inclusion.

Introduction. Soybean meal (SBM) has been successful as an alternative plant protein source of fishmeal due to its abundance, accessibility, affordable cost, and nutritional value (Akiyama 1991). It has the most well-balanced amino acid profile among plant protein ingredients. However, its production is inadequate in developing countries and the price has continued to rise due to increased global demand from animal as well as food industries and to unpredictable fluctuations in foreign exchange. Soybean meal being expensive, fish nutritionists have been trying to look for locally available and cheaper plant material to substitute for it in the diet of aquaculture species.

Ulva intestinalis is one of the green algae which could be an alternative ingredient in the diets of fish and shrimp. Highly invasive, it is one of the dominant macrophytes which can be found in nutrient-enriched marine or estuarine coastal environments. It forms nuisance blooms that cover benthic areas, litter beaches and disrupt littoral zone food webs by replacing indigenous algae and smothering aquatic animals (Sousa et al 2007). *Ulva* sp. contains 10-26% of crude protein content (Fleurence 1999) and all the essential amino acids (EAA) which account for 42.1-48.4% of the total amino acids content (Wong & Cheung 2000). It contains vitamins and minerals, especially rich in ascorbic acid (García-Casal et al 2007; Ortiz et al 2006).

Previously, we have evaluated the unprocessed form of this green seaweed as an ingredient in the diet of fry Nile tilapia (*Oreochromis niloticus*) (Aquino et al 2014). It promoted high survival and very good feed conversion ratios and the seaweed meal could replace 15% by weight of soybean meal without negatively affecting the growth, feed efficiency and body composition of the fish. This study aims to evaluate the optimum level of replacement of soybean meal with *U. intestinalis* protein concentrate in the diet of Nile tilapia.

Material and Method

Preparation of U. intestinalis protein concentrate. The feeding trial was conducted in the period between September to December 2013. *U. intestinalis* was collected from brackishwater ponds around Iloilo as described previously (Aquino et al 2014). Seaweeds were transported to the Institute of Aquaculture Multi-Species Hatchery, University of the Philippines Visayas, Miagao, Iloilo and were manually washed with freshwater, shade-dried for 3 to 4 days and oven-dried. Protein concentrate of the seaweed was prepared following the classical method of Agbede et al (2008) modified by including an acidification stage. Dried seaweeds were homogenized with distilled water using a handheld blender. The slurry was acidified by adding HCl to pH 2.0, squeezed in muslin cloth to collect the juice containing the protein. The juice was heated to 80-90°C for 10 min and the coagulated protein was separated out by filtering through a muslin cloth. The thick protein concentrate slurry was oven-dried to about 10% moisture and was kept at -20^{o}C until diet preparation.

Diet preparation. Four diets were formulated to provide 37% crude protein and 9% crude fat (Santiago et al 1982) and diet without the *Ulva* protein concentrate (UPC) served as the control diet. The UPC replaced soybean meal by weight at 0, 15, 30, and 45% (C, D1, D2, and D3 respectively (Table 1) equivalent to protein replacement of 0, 6, 12, and 18%, respectively. Feed ingredients were ground and thoroughly mixed with the addition of oil and a vitamin/mineral premix using a laboratory mixer. Gelatinized cornstarch was added last. The moistened mixture was pelleted (2 mm) in a meat grinder and oven dried (60^{o}C) for 4 to 6 h. Diets were crumbled into appropriate sizes (0.5 to 3.0 mm), sealed in plastic bags, and stored at -20^{o}C until use. The experimental diets were subjected to proximate analysis prior to the feeding experiment.

Table 1

Composition and proximate analyses of experimental diets of Nile tilapia fry for the evaluation of *U. intestinalis* protein concentrate (UPC) as a replacement for soybean meal in g kg^{-1}

Ingredients	*C (0%)*	*D1 (15%)*	*D2 (30%)*	*D3 (45%)*
Sardines meal	310.0	310.0	310.0	310.0
Soybean meal	260.0	221.0	182.0	143.0
Seaweeds UPC	0.0	39.0	78.0	117.0
Copra Meal	74.8	74.8	74.8	74.8
Rice Bran	120.9	120.9	120.9	120.9
Ipil-ipil leaf meal	101.0	101.0	101.0	101.0
Cod liver oil	30.0	30.0	30.0	30.0
Vegetable oil	20.0	20.0	20.0	20.0
Vitamin mix[a]	21.7	21.7	21.7	21.7
Mineral Mix[b]	21.6	21.6	21.6	21.6
Cornstarch	30.0	30.0	30.0	30.0
CMC	10.0	10.0	10.0	10.0
TOTAL	1000.0	1000.0	1000.0	1000.0
Proximate composition (dry weight basis)				
Dry matter	949.2	950.7	949.8	947.4
Crude protein	400.2	375.1	375.3	382.2
Crude fat	99.4	98.5	96.3	95.5
Crude fiber	64.7	62.4	61.5	62.7
Ash	140.5	150.2	164.0	183.2
NFE	295.2	313.9	302.8	276.3
Energy	3675.8	3642.0	3579.6	3493.9
TOTAL	1000.0	1000.0	1000.0	1000.0

a - Vitamin premix (kg^{-1} of diet): Vitamin A, 15600 IU; Vitamin D3, 2600 IU; Vitamin E, 260 IU; Vitamin B1, 104 mg; Vitamin B2, 104 mg; Vitamin B6, 65 mg; Vitamin B12, 26 µg; Niacin, 520 mg; Calcium pantothenate, 260 mg; Biotin, 0.52 mg; Folic acid, 23.4 mg; Ethoxyquin, 6.5 mg; b - Mineral premix (kg^{-1} of diet): Iron, 1200 mg; Manganese, 300 mg; Zinc, 1200 mg; Copper, 120 mg; Iodine, 54 mg; Cobalt, 600 µg; Selenium, 6 mg.

Feeding trial. Four thousand Nile tilapia fry were procured from the Southeast Asian Fisheries Development Council-Aquaculture Department in Tigbauan, Iloilo, Philippines, and acclimatized in a 1 ton capacity fiber glass tank with continuous aeration for 10 days and fed with the control diet. Two hundred seventy Nile tilapia fry were randomly stocked in eighteen 60-L tanks (15 fry $tank^{-1}$) and the remainder of the fish were sacrificed for initial proximate analysis. The experimental diets were fed to three replicate groups of Nile tilapia fry (0.03 ± 0.00 g) for 90 days. The trials were conducted in closed recirculating systems where filtered water from reservoir entered and left each tank at the rate of 1.3 L min^{-1}. Fifty to 70% of the water system volume were replaced every 2 days. Sampling was done every 10 days in which fish were batch-weighed which served as the basis for adjustment of the amount of feed in the next 10 days. Uneaten feeds and faeces were siphoned off every morning before the first feeding.

Water was subjected to chlorination (100 ppm NaClO) and dechlorination 3 days before use. Water temperature and pH were measured twice a day (08:00 and 16:00 h) while dissolved oxygen was measured twice a week, and nitrite and total ammonia weekly using commercially available kits (AQUA-NITE™ and AQUA- AM™, respectively).

Growth performance parameters. Growth and feed utilization efficiency were calculated using the following formulas:

$$\text{Weight gain, WG (g)} = W_2 - W_1$$

$$\text{Specific Growth Rate (SGR, \% day}^{-1}) = \frac{(\ln W_2 - \ln W_1)}{(T_2 - T_1)} \times 100$$

$$\text{FCR} = \text{Feed intake (g)/Weight gain (g)}$$

$$\text{Nutrient Retention (\%)} = (\text{\% final carcass nutrient} \times \text{final ABW (g)}) - (\text{\% initial carcass nutrient} \times \text{initial ABW (g)})] \times 100 / \text{total nutrient intake (g)}$$

Where,

W_2 = Final weight (g)
W_1 = Initial weight (g)
T_2 = Final time (in days)
T_1 = Initial time (in days)
ABW = average body weight (g)

Carcass analysis. Three thousand seven hundred thirty fish were taken at the start of the experiment and kept frozen (-20°C) for the proximate analysis of initial carcass composition. At the end of the feeding trial, fish from each tank were pooled by treatment, weighed, dried and subjected to final carcass analysis. Moisture was measured using a thermo-balance (Mettler 32 Toledo HB43 halogen moisture analyzer). 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 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.

Statistical analysis. Statistical computations were done with Statistical Package for Social Sciences (SPSS) version 16.0 for Windows. Growth and feed efficiency data were tested for homogeneity of variance and normality of data using Levene's and Shapiro-Wilk *W* tests before they were subjected to one-way analysis of variance (ANOVA) at 0.05 significance. Means of parameters (WG, SGR, FI, FCR, nutrient retentions and survival rate) among experimental groups were compared using Tukey's HSD test.

Results and Discussion

Proximate composition of UPC and unprocessed meal. Table 2 shows the proximate composition of the UPC and the unprocessed seaweed meal. Similar to the findings of Peña-Rodríguez et al (2011) in cultivated *Ulva clathrata*, ashes, fibre and protein of *U. intestinalis* were the most abundant in the unconcentrated seaweed meal. Crude protein content was similar to that obtained by Ergun et al (2009) also in *U. intestinalis*. However, the levels of crude protein (9.9%) and crude lipid (1.43%) were lower in the present study than the reported values of Peña-Rodríguez et al (2011) (averages of 23.6% and 3.0%, respectively) while ash content in the present study (59.0%) was higher than their average content of 46.7%. The differences in crude protein content might be largely due to the N fertilization of the cultivated *U. clathrata* in their study while *U. intestinalis* used in the present study was from natural stocks. The wash step used in the studies also could have made a difference in eliminating ash content such that if the protein content was expressed on an ash free dry basis (assuming that all the N measured absolutely came from protein), it amounted to 24.2% in the present study. In the process of concentrating protein from the green seaweed, fiber was reduced by 23.2% (from 59.0% to 13.7%) but the protein content was tripled (increased by 31.3%) to 31.6%, while the ash content was minimally reduced by about 1.0% only.

Table 2

Proximate analysis of *U. intestinalis* protein concentrate and unprocessed meal in dry weight basis (g kg^{-1})

Chemical composition	*U. intestinalis protein concentrate*	*Unprocessed U. intestinalis meal*
Dry matter	907.0	837.8
Crude protein	316.3	99.1
Crude lipid	276.0	143.0
Crude fibre	13.7	59.0
Ash	314.2	330.5

Studies with low protein levels similar to the present study but either lower or similar values for ashes have been reported (5.9-17% and 17.5-55% dw, respectively) for the natural stocks of *U. fasciata* and *U. intestinalis* (McDermid & Stuercke 2003), *U. lactuca* (Wong & Cheung 2000) and for *E. intestinalis* and *Enteromorpha* sp. in Mexico (Aguilera-Morales et al 2005). Results from other studies show higher protein but lower ash values (21.1-29.5% and 11-29% dw, respectively) for *U. lactuca* cultivated in Spain (Ventura & Castañón 1998), *U. rigida* from the Portuguese coast (Valente et al 2006), *U. lactuca* from the coastal area of Northern Chile (Ortiz et al 2006), from Holbeck, UK (Marsham et al 2007) and for *Enteromorpha prolifera*, *E. linza* and *U. fasciata* cultivated in India (13.4-22.6 and 6.6-35.6% dw, respectively; Naidu et al 1993). In general, protein and ash values reported for wild Ulvales are inversely correlated, and have wider variations compared with those of cultivated seaweeds which according to Peña-Rodríguez et al (2011) seemed related to the degree of variation in environmental conditions. A high inorganic content of seaweeds is very common and it is due to the ability of seaweeds to deposit elements present in the water medium where they live (Chapman & Chapman 1980).

Growth performance. Comparison of results in previous replacement studies with the present study is difficult since most, if not all, of the replacement studies have used unprocessed meal of seaweeds rather than protein concentrates (PCs). Bearing in mind that the main difference between the unprocessed meal and the PC was the reduction of crude fiber and a considerable increase in crude protein with concomitant slight decrease in ash, comparison could still be made. In the present study, weight gain (WG) and specific growth rate (SGR) of fish fed the control diet were statistically similar with those of fish fed diet containing 15% UPC replacement; those of fish fed diets containing 30 and 45% UPC replacements were significantly lower (Table 3). Azaza et al (2008) have

conducted replacement study of soybean meal with the unprocessed *U. rigida* meal and have concluded that the seaweed meal could constitute up to 20% of the Nile tilapia diet without any depressive effect on fish growth and feed utilization efficiency; beyond 20% inclusion, fish show decreasing growth. Guroy et al (2007) have observed that inclusion of 10-15% *U. rigida* meal to the diet of the Nile tilapia was optimum for growth performance. Ergun et al (2008) have observed that inclusion of 5% *U. rigida* meal was optimum for the Nile tilapia juvenile. Diler et al (2007) have attempted to replace wheat meal with unprocessed *U. rigida* meal in the common carp (*Cyprinus carpio*); the two protein sources are quite similar in protein content i.e. 8.63 and 8.00%, respectively. Their results were similar to those of the present study in which the WG is statistically similar in the control and that of 15% replacement group. The comparison is interesting since both the Nile tilapia and the common carp are both omnivorous. In black sea bream (*Acanthopagrus schlegeli*), red sea bream (*Pagrus major*), rainbow trout (*Oncorhynchus mykiss*) (Mustafa & Nakagawa 1995), supplementation of *U. rigida* in the range of 5 to 15% to the diet not only improves the growth performance but also the quality of carp as a protein product.

Table 3

Growth performance, feed and nutrient utilization of Nile tilapia fed with *U. intestinalis* protein concentrate (UPC) diets for 90 days

Parameters	*Diets*			
	C	*D1 (15%)*	*D2 (30%)*	*D3 (45%)*
Weight gain (g)	7.00 ± 0.55^{a}	5.37 ± 0.38^{ab}	4.67 ± 0.43^{b}	4.20 ± 0.31^{b}
SGR (% day^{-1})	6.79 ± 0.10^{a}	6.48 ± 0.09^{ab}	6.30 ± 0.11^{b}	6.18 ± 0.09^{b}
Feed intake (g fish^{-1})	6.92 ± 0.48^{a}	5.60 ± 0.18^{b}	4.79 ± 0.24^{bc}	4.20 ± 0.14^{c}
FCR	1.00±0.02	1.05±0.04	1.01±0.04	1.02±0.05
Survival rate (%)	95.6±2.2	88.9±2.2	91.1±2.2	91.1±2.2
Protein retention (%)	29.6±0.5	29.6±1.2	29.0±1.2	28.2±1.5
Lipid retention (%)	9.59 ± 0.16^{a}	7.36 ± 0.29^{b}	7.58 ± 0.31^{b}	7.52 ± 0.40^{b}

SGR - specific growth rate; FCR - feed conversion ratio. Values are expressed as means ± S.E.M. Means in the same row with different superscripts are significantly different ($p < 0.05$).

Fish accepted their respective diets and fed aggressively in the present study. Significantly lower feed intake was observed in fish fed the diet containing 30 and 45% of UPC. Fish fed the control diet exhibited significantly the highest feed intake followed by those fed diets with 15% UPC. Although restricted feeding was done in the present study and not apparent satiation, it was made sure that the feed allocation was fine-tuned daily especially if the fish were observed unable to consume the day's allocation; uneaten feed were estimated daily. Since there was a trend of decreasing feed intake with increasing inclusion of UPC, it was possible that palatability was a factor. It could be that in the process of concentrating the protein component of *U. intestinalis*, 'unpalatable' components might have also been concentrated. Azaza et al (2008) have have measured the antinutrients of *U. rigida*; 30 % *U. rigida* meal contains on a dry weight basis 2.65% saponins, 0.22% tannins and 0.61% phytic acid. The saponin content in the diet may not necessarily result in the negative performance as we have shown in the common carp previously (Serrano 2013) but needs validation in the future. Azaza et al (2008) have observed that the feed intake of the Nile tilapia is high at 30% inclusion of the unprocessed *U. rigida* meal and explained this as a result of the lower digestible energy inducing higher feed intake. We have measured previously the Apparent Digestibility of Ingredient (ADI) in *Penaeus monodon* to be 99.13% (Santizo et al 2014) and *U. intestinalis* protein concentrate to be 94.7% (unpubl. data), the digestible energy content of the UPC could not be a factor that influenced feed intake in the present study.

Feed efficiency (i.e. food conversion ratio, FCR), survival and protein retention in the present study were independent of the dietary treatments. This is in contrast to the results of feeding 15% *U. rigida* to common carp which resulted in higher protein retention than did fish fed the control diet (Diler et al 2007). Lipid retention of fish fed

the control diet in the present study was significantly higher than those fed with other diets which was in contrast to the results of Nakagawa et al (1993) in the carnivorous black sea bream which accumulated intraperitonal fat when fed the algae meal supplemented diets more efficiently than did fish fed the control diet.

Table 4 shows that body crude lipid were higher in the final carcass of tilapia than in the initial fry while body crude protein and ash were lower although comparison was numerical because the analyses were for pooled samples for each treatment. The observation of Azaza et al (2008) of an overall trend of decreasing carcass lipid content of the Nile tilapia with increasing inclusion levels of *U. rigida* meal and also of constant crude protein and ash content were not observed in the present study. Similar to the findings of Guroy et al (2007) moisture and ash content of fish were not significantly affected by the dietary supplementation of UPC.

Table 4

Body composition of Nile tilapia fed with *U. intestinalis* protein concentrate (UPC) diets at the beginning and end of experiment

Component (g kg^{-1})	*Initial*	*Final (fish fed diet)*			
		C	*D1 (15%)*	*D2 (30%)*	*D3 (45%)*
Dry matter	927.1	954.0	950.8	948.0	945.1
Crude protein	672.3	630.4	667.8	652.6	655.5
Crude lipid	134.2	204.2	166.0	170.3	174.9
Ash	177.1	163.1	163.9	170.6	166.2

Conclusions. The present study demonstrated that the protein concentrate of *U. intestinalis* produced from the combined heat and acidification processes was a potential feed ingredient for Nile tilapia. It could replace up to 15% of the soybean meal of the diet without adversely affecting specific growth rate, feed utilization efficiency and survival but with possible slight reduction in feed intake and body crude lipid.

Acknowledgements. The authors wish to express their gratitude to the Philippine Department of Science and Technology (DOST) Philippine Council for Agriculture, Aquatic and Natural Resources Development (PCAARD) for the funding and DOST-Accelerated Science and Technology Human Resource Development Program (ASTHRDP) for the scholarship provided. They also wish to thank the UPV Office of the Research and Extension for the additional funding and publication support. They are also grateful to Dr. Rex Ferdinand M. Traifalgar for his technical support.

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Received: 04 July 2014. Accepted: 02 August 2014. Published online: 03 August 2014.
Authors:
Augusto Erum Serrano Jr., University of the Philippines Visayas, College of Fisheries and Ocean Sciences, Institute of Aquaculture, Philippines, Miagao, Iloilo 5023, e-mail: serrano.gus@gmail.com
Jon Irish Legaspi Aquino, Southeast Asian Fisheries Development Council-Aquaculture Department, Tigbauan, Iloilo, Philippines, e-mail: perfectmage01@gmail.com

How to cite this article:
Serrano Jr. A. E., Aquino J. I. L., 2014 Protein concentrate of *Ulva intestinalis* (Chlorophyta, Ulvaceae) could replace soybean meal in the diet of *Oreochromis niloticus* fry. AACL Bioflux 7(4):255-262.