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

Composition, chemical score (CS) and essential amino acid index (EAAI) of the crinkle grass *Rhizoclonium* sp. as ingredient for aquafeeds

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Abstract. Biochemical analyses were done on the crinkle grass *Rhizoclonium* sp. to evaluate its nutritive value for use in aquafeeds. Crinkle grass were collected from two brackish water sources (Leganes and Arevalo both in the Iloilo Province, Philippines). Regardless of collection site, proximate analyses showed that crude protein, fiber and ash were in the ranges measured for other macroalgae; crude fat values, in contrast, were lower. As for the amino acid composition, aspartic and glutamic acids (13 and 15%, respectively) exhibited the highest proportion but were lower than the values of 26 and 32%, respectively, measured in other green seaweeds. The Arevalo seaweed exhibited higher essential amino acid content (EAA, 31.1 g 100 g protein^{-1}) than in those from Leganes (48.3 g 100 g protein^{-1}). The ratio of EAA to non-essential AA was higher in the Arevalo seaweed (1.11:1) than that in the Leganes seaweed. The EAA index (EAAI) of the seaweed from either site for the Nile tilapia and for the tiger prawn exhibited similar values. The first and second limiting amino acid in Leganes *Rhizoclonium* for the Nile tilapia was Tryptophan (Trp) and Lysine (Lys) while the Arevalo seaweed were Methionine (Met) and Trpt for the first and second limiting AA, respectively. For the tiger shrimp, Leganes *Rhizoclonium* exhibited Phenylalanine (Phe) and Lys, while the Arevalo seaweed were Met and Phe (first and second limiting EAA). All the other amino acids (7 out of 10 in seaweeds from Leganes and 6 out of 10 in those from Arevalo) were either higher than or equal to their EAA requirements. The chemical scores (CS) of the seaweed were similar for the Nile tilapia (62.2 and 62.0, respectively) for the Leganes and Arevalo *Rhizoclonium*. For *Penaeus monodon*, the CS of the seaweed was similar with that of the Leganes seaweed (64.3) but was markedly lower (41.9) in the Arevalo seaweed. Conclusions: Proximate composition of *Rhizoclonium* sp. varied slightly with the sampling site. Chemical scores could be similar or variable, but the kind of limiting amino acids varied. *Rhizoclonium* sp. regardless of habitat exhibited EAAI around 1.0 pointing to a well-balanced AA content. All the determined parameters pointed to the possibility of incorporating the seaweed and supplementing other protein components in the diets of the Nile tilapia and in the tiger prawn.
Key Words: nutritive value, macroalgae, proximate analysis, amino acid profile.

Introduction. An alternative cheap ingredient in the diets of aquaculture species is a necessity in order for aquaculture to fulfill its role of providing proteins for humans, replacing those that come from captures fisheries. For decades, carcass amino acid profile of aquaculture species has been used as a guide in formulating an effective and least-cost diet (Deshimaru & Shigueno 1972; Cowey & Tacon 1983; Wilson & Poe 1985) to predict the dietary protein requirement of the animal. At present, this approach could still be used for nutritive evaluation of certain protein sources with the use of whole chicken egg as a standard protein (WHO/FAO/UNU 1985). This is because no specific species is yet in the mind of the investigator and the whole approach is to evaluate a protein source to be applied to any animal whether aquatic, livestock or human. Currently however, the essential amino acid (EAA) requirements of some aquaculture species have already been established including those of the Nile tilapia *Oreochromis niloticus* and the tiger prawn *Penaeus monodon* (Santiago & Lovell 1988; Akiyama 1992). Thus, the amino acid profile of a candidate protein source could be used to evaluate its nutritive value and could be made specific to the Nile tilapia or the tiger prawn by using

their established EAA requirement profiles as references instead of that of the whole chicken egg.

Proximate composition provides information about the major nutrient and gross energy contents of feeds and feedstuffs (Jobling 2001). The idea is for the required major nutrients (protein, fat and carbohydrate) to be fixed and replacements of ingredients can be done in the formulation. Proximate analysis is used as a rough and initial evaluation of feeds and feedstuffs. Another evaluation criteria is the protein chemical score (CS) defined as the lowest ratio of the essential amino acid content in the test protein to the content of each amino acid in the muscle protein or to the EAA required level when the EAA requirement is already established. The assumption of CS was that whole egg protein is of the highest biological value (BV) and therefore the most suitable for growth, and that growth will be limited by that essential amino acid in the diet whose ratio to its content in the whole egg protein is the lowest (Hepher 1988). Although the first limiting amino acid has an important role in determining the relative value of the dietary protein, it was realized that other essential amino acids may also have some effect on it. This resulted in the development of the 'essential amino acid index' (EAAI). It is the geometrical mean of the ratio of all EAA in the evaluated protein relative to their content in a highly nutritive reference protein such as whole egg (Oser 1959). Chemical score and EAAI were developed mainly for evaluating proteins in diets of farm animals (Hepher 1988). In using those quotients for evaluating proteins in fish diets it is assumed that chicken egg protein is also the best for fish. Until more information is available, the CS and EAAI may serve as practical means for evaluating protein in fish diets. The comparison of the amino acid composition of the evaluated protein to any reference of 'best' protein such as egg protein is relevant only as long as the actual requirements for amino acids by the fish are not known. The requirements for amino acids have been established for a number of species. These requirements may be better references for evaluating dietary proteins than egg protein (Hepher 1988).

Animal protein sources used in the diets of the Nile tilapia and of the tiger prawn are expensive because majority of the ingredients are imported, hence, cheap and locally available substitutes should be investigated. Macroalgae are good candidates to replace the imported soybean meal in aquafeeds. A relatively high content of proteins are found in red and green seaweed, consisting on average 10–30% of their dry matter (Ramos et al 2000). This amount of protein is comparable with the amount of protein in soya-bean (Galland-Irmouli et al 1999; Burtin 2003). *Rhizoclonium* spp. is a green macroalgae that belongs to the Ulvophytes that are diverse morphologically and ecologically (Berger & Kaever 1992). The group is predominantly marine and includes the sea lettuce *Ulva* (Hayden & Waaland 2002). *Rhizoclonium riparium* can grow year round with specific growth rate range from 2.1 to 10.4% per day; optimal salinity and temperature are 20 ppt and 25^{o}C, respectively (Chao et al 2005). It has a very wide salinity tolerance which ranges from 0.1 to 34 ‰ (Imai et al 1997). The use of natural filamentous algal biomass such as *Rhizoclonium* spp. can also be possible to apply as formulated feed only because they clog the gills of the fishes in natural ponds, hence, always being avoided by the fish fauna (Roy et al 2011).

This study was undertaken to assess the potential of the chlorophyte macroalgae *Rhizoclonium* spp. as a partial protein substitute to diets of the Nile tilapia and tiger prawn using proximate composition, CS and the EAAI with their established EAA requirements as the reference profile.

Material and method

***Collection and preparation of Rhizoclonium* meal**. *Rhizoclonium* sp. was collected from two different sites: Leganes, Iloilo and Villa Arevalo, Iloilo. The seaweed was immediately transported to the laboratory for preparation. The collected algae were air dried for 48 h, oven dried for 24 h at 60°C and were pulverized using a mechanical grinder and then passed through a 150 µm sieve. The powdered *Rhizoclonium* sp. (from here on referred to as *Rhizoclonium* meal) was then stored at -20°C.

Proximate analysis. A sample of the *Rhizoclonium* meal was subjected to proximate analysis, following standard protocol (AOAC 1996). This included analysis of the following chemical compounds: moisture, ash, crude protein, crude fat, crude fiber, and nitrogen-free extracts (digestible carbohydrates). All analysis was done in 3 replicates. Moisture (%) was analyzed using a Mettler-Toledo® Moisture Analyzer. Two g of the sample was weighed, placed in an air oven at 135 ± 2°C and dried for 2 h to constant weight. The loss in weight was considered as the moisture content of the meal. Ash was analyzed by weighing 2 g of the sample into a porcelain crucible and was placed in a temperature controlled furnace at 600°C for 2 h and weighed. Crude protein was determined using Foss® Kjeltec 2300. One g of the sample was digested in the presence of sulfuric acid, potassium sulfate, and copper sulfate. Sodium hydroxide was added into the digested solution, which was distilled and titrated with a standard acid solution. The crude protein was calculated as Kjeldahl Nitrogen multiplied with the factor of 6.25. Determination of crude fat was done using Foss® Soxtec 2055. Crude fat from 2 g of sample was extracted with anhydrous ether and the resulting extract was dried at 100°C to constant weight. The loss in weight was reported as ether extract or crude fat. Crude fiber was analyzed using Foss® Fibertec M6. Two g of the sample was weighed and boiled in a sulfuric acid solution. The resulting residue was rinsed and filtered, then boiled again in a sodium hydroxide solution. The resulting residue was rinsed and filtered again, transferred to a crucible, dried overnight at 110°C to a constant weight. This was then ashed for 2 h at 550 ± 10° and weighed. Nitrogen free extract representing the soluble carbohydrates in the sample was computed by difference.

Amino acid profile. A sample of the *Rhizoclonium* meal was sent to SGS Philippines for amino acid profiling, which included analysis of the following essential and non-essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, serine, aspartic acid, glutamic acid, arginine, glycine, alanine, proline, cysteine, and tyrosine. The protocol for the analysis of the amino acids followed AOAC (1996) using Waters® High Performance Liquid Chromatography (HPLC/Fluorescence Model).

Analytical methods and calculations. Amino acid results were expressed as g 100 g^{-1} protein. The essential amino acid (A/E) ratio (Arai 1981) of each essential amino acid (EAA) was calculated as the percentage of the total EAA. The chemical score of the *Rhizoclonium* meal was determined by the following formula:

Chemical score = % limiting EAA in *Rhizoclonium* sp. / % corresponding required EAA levels in the Nile tilapia or in the tiger prawn

The EAAI of *Rhizoclonium* meal in the two species was determined from the formula:

$$EAAI = 10^{\log EAA}$$

Where: $\log EAA = 0.1[\log(a^1/a_{1s} \times 100) + \log(a_2/a_{2s} \times 100) + \log(a_n/a_{ns} \times 100)]$;
$a_1..$, a_n are the amino acid contents of *Rhizoclonium* sp. while $a_{1s}...a_{ns}$ are the required levels of these amino acids in the Nile tilapia or in the tiger prawn.

The geometric mean, EAAI, is the ratio of all essential amino acids of *Rhizoclonium* meal to those of chicken egg (Oser 1959).

Results and discussion

Rhizoclonium spp. meal composition. Proximate composition of *Rhizoclonium* sp. varied with sample site (Table 1). All the major classes of nutrients in the seaweed collected from Leganes significantly differed from that collected from Arevalo except ash which exhibited the highest proportion among major biochemical components. Ash and crude fiber content of the seaweed from the two sites were half or more (55.5% from

Leganes and 50% from Arevalo) of the total dry matter of the seaweed. The range of crude protein, crude lipid, ash and fiber in *Rhizoclonium* seaweed in the present study ranged from 13.93 to 15.55%, from 0.21 to 0.56%, from 35.57 to 35.72% and from 17.70 to 19.77%, respectively. Cruz-Suarez et al (2008) measured the same components for green seaweed meals and reported values from 7 to 29, from 0.5 to 4, from 13 to 36 and from 3 to 6%, respectively. The difference in the results of the present study from those of Cruz-Suarez et al (2008) was a slightly lower lipid content and very high fiber content. *Rhizoclonium* meal contained high ash of 36% dry weight similar to the values reported for *Ulva intestinalis* (33%, Aquino et al 2014) and *Ulva lactuca* (31.7%, (Santizo et al 2014). These values were lower than those reported by other authors (Pak & Araya 1992, 1996a,b; Ortiz et al 2006).

The crude protein contents (15.55 and 13.93% dry basis, for those taken from Leganes and Arevalo, respectively; Table 4) of the *Rhizoclonium* meal in the present study (Table 1) were in agreement with values reported for various macroalgae (Murthy & Radia 1978; El-Tawil & Khalil 1983; Zavodnik 1987; Santizo et al 2014) of 13.6–24.5% dry weight. Chemical analyses of various filamentous algae and seaweeds shows that filamentous algae contain about 16-32 % protein while red and green seaweeds have varying protein content, ranging between 3-29 % and 6-26 %, respectively (Hasan & Chakrabarti 2010). The spatial variation in the protein level of *Rhizoclonium* sp. could be attributed to the difference in nutrient level (preferably nitrate) of the collection sites as hypothesized by Aberjeen et al (2009). Furthermore, the chemical composition of seaweeds is influenced by factors, in addition to species, like habitat, maturity, geographical locations and seasonal (Ito & Hori 1989; Fleurence 1999).

It has been established that seaweeds in general contain 4% fat (Herbetreau et al 1997). The fat content of *Rhizoclonium* sp. in the present study (0.21 and 0.56% dry weight for Leganes and Arevalo seaweed, respectively; Table 1) were lower than the average value of seaweeds (Cruz-Suarez et al 2008). Ortiz et al (2006) hypothesize that the differences in fat content could be due to factors such as geography of development of the seaweed.

Table 1

Proximate composition of *Rhizoclonium* sp. from the two sites: Leganes and Arevalo

Proximate composition (%)	*Sample site*		*P (a=0.05)**
	Leganes	Arevalo	
Moisture	7.42 ± 0.05	12.25 ± 0.17	0.00
Ash	35.72 ± 0.09	35.57 ± 0.47	0.73
Crude protein	15.55 ± 0.11	13.93 ± 0.22	0.01
Crude fat	0.21 ± 0.04	0.56 ± 0.08	0.02
Crude fiber	19.77 ± 0.25	17.70 ± 0.11	0.03
Nitrogen free extract	28.75 ± 0.31	32.23 ± 0.51	0.03

*Student t test. Values are expressed as mean ± SEM.

Seaweeds are known as an excellent source of vitamins and minerals, especially sodium and iodine (Ortiz et al 2006) due to their high polysaccharide content implying that soluble and insoluble dietary fiber are very high. The crude fiber content in the present study was lower than those reported in other macroalgae (Ortiz et al 2006; Aquino et al 2014; Santizo et al 2014). Nevertheless, the ash content in the present study coincided with the range suggested for seaweeds in general *i.e.* 8 to 40% of algal dry weight (Rupérez 2002).

Table 2

Amino acid (AA) profile of *Rhizoclonium* meal from the two sites: Leganes and Arevalo

AA Content (% crude protein)	*Leganes*	*Arevalo*
Essential AAs		
Phenylalanine	3.55	6.20
Valine	4.93	7.42
Tryptophan	0.51	0.94
Threonine	2.75	5.05
Isoleucine	3.51	5.30
Methionine	1.37	1.77
Histidine	1.43	1.89
Arginine	4.46	5.54
Leucine	5.46	8.53
Lysine	3.14	5.68
Nonessential AAs		
Tyrosine	1.67	2.18
Serine	0.39	0.39
Aspartic acid	9.62	8.20
Glutamic Acid	11.52	12.89
Glycine	5.30	7.02
Alanine	5.34	6.67
Proline	3.78	4.61
Cysteine	0.56	0.70
Total	69.29	90.98
EAA/NEAA	0.8:1	1.1:1

Amino acid composition. All essential amino acids were present in *Rhizoclonium* sp. regardless of collection site (Table 2). As was observed in most seaweed, aspartic and glutamic acid constituted together a large part of the amino acid fraction. In the green seaweeds, these two amino acids can represent between 26 and 32% (Cruz-Suarez et al 2008). In the present study, these values were lower, from 13.10-13.21 to 15.02-15.22%, respectively. The EAA concentrations of the seaweed were higher in those collected from Arevalo (31.11 per 100 g crude protein) than in those from Leganes (48.32 g per 100 g crude protein). The ratio of EAA to NEAA was higher in seaweed from Arevalo (1.1:1) than that from Leganes (0.8:1). The essential amino acids Arg, His, Leu, Phe, Met and Val were higher in the seaweeds from Leganes (Table 3) than their corresponding EAA required levels in the Nile tilapia. In *Rhizoclonium* from Arevalo was the same except Met was the lowest while Thr exceeded the required level in the Nile tilapia. For the tiger prawn, *Rhizoclonium* collected from Leganes exhibited higher Iso, Leu and Val than the levels needed by the Penaeid shrimp; however, those collected from Arevalo exhibited higher Leu, Thr, Trp and Val. It appeared that the A/E ratio varied with the collection site.

The CS of *Rhizoclonium* meal for the Nile tilapia was similar (61.2 and 62, Table 3 & 4) regardless of collection site. However, the CS of the seaweed for the tiger shrimp were 64.3 in those collected from Leganes and 41.9 in those from Arevalo. The first and second limiting amino acid in Leganes *Rhizoclonium* for the Nile tilapia was Trp and Lys while the Arevalo seaweed were Met and Trp, respectively. For the tiger shrimp, the first and second limiting AA in Leganes *Rhizoclonium* was Phe and Lys, respectively, while in the Arevalo seaweed was Met and Phe, respectively. All the other amino acids (7 out of 10 in seaweeds from Leganes and 6 out of 10 in those from Arevalo) were either higher than or equal to their EAA requirements. It is evident that *Rhizoclonium* could be incorporated into aquaculture feed formulations, specifically for the Nile tilapia and the tiger prawn.

Table 3

Essential amino acid index (EAAI), chemical score (CS) and A/E ratios of *Rhizoclonium* meal collected from Leganes, Iloilo and EAA requirement levels for the Nile tilapia and the tiger prawn

Specification	*Rhizoclonium* [*1]	*Tilapia req.* [*2]	*Penaeid req.* [*3]	*A/E* [*4]			*(A/E Rhizoclonium)/(A/E req.)(%)*	
	AA (% CP)			*Rhizoclonium meal*	*Nile tilapia*	*Penaeid shrimp*	*Nile tilapia*	*Penaeid shrimp*
Arginine	4.46	4.20	5.8	13.92	10.6	14.08	131.3	98.9
Histidine	1.43	1.72	2.1	4.46	4.2	5.10	106.2	87.5
Isoleucine	3.51	3.11	3.5	10.96	11.0	8.50	99.6	128.9
Leucine	5.46	3.39	5.4	17.04	15.4	13.11	110.7	130.0
Lysine	3.14	5.12	5.3	9.80	12.2	12.86	80.3	76.2
Phenylalanine	3.55	2.68	7.1	11.08	10.0	17.23	110.8	64.3
Methionine	2.30	3.75	3.6	7.18	5.9	8.74	121.7	82.2
Threonine	2.75	3.75	3.6	8.58	8.9	8.74	96.4	98.2
Tryptophan	0.51	1.00	0.8	1.59	2.6	1.94	61.2	82.0
Valine	4.93	2.80	4.0	15.39	11.9	9.71	129.3	158.5
Total	32.04	31.52	41.2	-	-	-	-	-
EAAI	-	-	-	-	1.02	0.97	-	-
CS	-	-	-	-	61.2	64.3	-	-

*[1]Values are averages of the EAA of *Rhizoclonium* sp. collected from the two sites
*[2] Santiago & Lovell (1988)
*[3] Akiyama (1992)
*[4] A/E is the ratio of a given essential amino acid to the total essential amino acids
AA – amino acid
CP – crude proteine

Table 4

Essential amino acid index (EAAI), chemical score (CS) and A/E ratios of *Rhizoclonium* meal collected from Arevalo, Iloilo and EAA requirement levels for the Nile tilapia and the tiger prawn

Specification	*Rhizoclonium* [*1]	*Tilapia req.* [*2]	*Penaeid req.* [*3]	*A/E*[*4]			*(A/E Rhizoclonium)/(A/E req.) (%)*	
	AA (% CP)			*Rhizoclonium meal*	*Nile tilapia*	*Penaeid shrimp*	*Nile tilapia*	*Penaeid shrimp*
Arginine	5.54	4.20	5.8	11.47	10.6	14.08	108.2	81.5
Histidine	1.89	1.72	2.1	3.91	4.2	5.10	93.1	76.7
Isoleucine	5.30	3.11	3.5	10.97	11.0	8.50	99.7	99.7
Leucine	8.53	3.39	5.4	17.65	15.4	13.11	114.6	134.6
Lysine	5.68	5.12	5.3	11.76	12.2	12.86	96.4	91.5
Phenylalanine	6.20	2.68	7.1	12.83	10.0	17.23	128.3	74.5
Methionine	1.77	3.75	3.6	3.66	5.9	8.74	62.0	41.9
Threonine	5.05	3.75	3.6	10.45	8.9	8.74	117.4	119.6
Tryptophan	0.94	1.00	0.8	1.95	2.6	1.94	75.0	100.5
Valine	7.42	2.80	4.0	15.36	11.9	9.71	129.1	158.2
Total	48.32	31.52	41.2	-	-	-	-	-
EAAI	-	-	-	-	1.00	0.92	-	-
CS	-	-	-	-	62.0	41.9	-	-

*[1]Values are averages of the EAA of *Rhizoclonium* sp. collected from the two sites
*[2] Santiago & Lovell (1988)
*[3] Akiyama (1992)
*[4] A/E is the ratio of a given essential amino acid to the total essential amino acids
AA – amino acid
CP – crude proteine

The EAAI index of the *Rhizoclonium* collected from Leganes in the present study for the Nile tilapia and the tiger prawn were estimated to be 1.02 and 0.97, respectively, while that collected from Arevalo were 1.00 and 0.92, respectively. Oser (1959) developed the EAAI criteria for protein quality of feedstuff which was later used by Peñaflorida (1989).

Good quality protein sources have AEEI of 0.90, useful protein 0.80 and incomplete protein 0.70. Based on this criterion, *Rhizoclonium* meal in the present study could be considered as a good quality protein source regardless of habitat, because it contained a balanced amount of EAAs. CSs and EAAI are acceptable indices of biological value specifically when they are utilized as single source of protein. This is the reason why animal nutritionists recommend combination of protein sources i.e. a protein source could be complementary with other protein sources resulting in a well balanced protein in terms of its AA content. However the two indices are good indicators for a very quick evaluation of protein source until the data concerning the biological value (the result of a complete feeding trial) is not available for the time being. They are useful until feeding trial.

Conclusions. Although the crude protein of *Rhizoclonium* sp. was lower than the 20% lower limit before an ingredient could be called a protein supplement, its amino acid profile indicated that it could provide a well-balanced essential and non-essential amino acids and could be considered as a good quality protein. It is evident that *Rhizoclonium* could be incorporated into aquaculture feed formulations, specifically for the Nile tilapia and the tiger prawn.

Acknowledgements. The authors are grateful to the University of the Philippines Visayas for providing the research fund through the Office of the Vice Chancellor for Research and Extension (OVCRE) and to the Philippine Department of Science and Technology (DOST) - Accelerated Science and Technology Human Resource Development Program (ASTHRDP) for providing the scholarship to Ms. Bunda.

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Received: 15 April 2015. Accepted: 09 June 2015. Published online: 15 June 2015.
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How to cite this article:
Bunda M. G. B., Tumbokon B. L. M., Serrano Jr. A. E., 2015 Composition, chemical score (CS) and essential amino acid index (EAAI) of the crinkle grass *Rhizoclonium* sp. as ingredient for aquafeeds. AACL Bioflux 8(3):411-420.