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# *In vitro* prediction of digestible protein content of marine microalgae (*Nannochloropsis granulata*) meals for Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Digestible protein (DP) contents of novel feed ingredients are required for test diet formulation and commercial feed production. Species-specific *in vitro* pH-Stat protein hydrolysis was used to predict the DP contents of three algal meals produced from a common lot of the marine eustigmatophyte microalga, *Nannochloropsis granulata*, for juvenile Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*). Protein degree of hydrolysis (DH) and predicted protein apparent digestibility coefficients (ADCs) for Pacific white shrimp were statistically similar for all meals with average DH of 2.09% ($P = 0.052$) and predicted ADC of 74.6% ($P = 0.053$). Alternatively, meals processed by supercritical fluid extraction at 70 and 90 °C showed significantly ($P < 0.001$) higher DH and predicted ADC than the untreated base material for rainbow trout with average DH of 4.79% and predicted ADC of 87.0%, compared to 2.53% and 79.1%, respectively. Predicted protein ADC for all *N. granulata* meals was moderate for Pacific white shrimp (69–78%) and high for rainbow trout (79–88%) and therefore indicates their potential for use in fish and shrimp diets. Based on our results, we suggest DP values (dry matter basis) for similar *N. granulata* meals of 26% for Pacific white shrimp and 29% for rainbow trout.


## 1. Introduction

Aquaculture is the fastest growing food production sector globally with a production of 101 million tonnes annually worth $166 billion USD. Farmed seafood currently provides >50% of all seafood consumed globally today and this proportion is projected to rise to 62% by 2030 [1]. With a strong push towards economic and ecological sustainability, this sector requires additional alternatives to conventional feed inputs [2]. As such, aquaculture is seen as one of the most promising feed sectors for valorization of algae-derived products, but studies that evaluate its inclusion in modern aquafeeds are just now beginning to emerge. *Nannochloropsis granulata* is a marine eustigmatophyte microalga that is relatively new in phytoplankton taxonomy; having been uniquely identified more recently than other more established *Nannochloropsis* species (namely *N. oculata* and *N. gaditana*). While closely-related, *N. granulata* differs from other species in this genus with respect to chloroplast structures and 18S rRNA gene sequence [3] and far less is known about its potential for industrial applications. Supercritical fluid extraction (SFE) is widely used in bioprocessing of aquatic and crop based resources for the production of valuable consumer products [4]. In particular, SFE employing $CO_2$ as a solvent is useful for extracting compounds from bulk microalgae biomass (*e.g.*, essential fatty acids, carotenoids, bioactive compounds, *etc.*) destined as food or feed due to the relatively benign extraction conditions and resultant solvent-free products [5]. In most cases, the targeted extraction products have a high economic value to justify the relatively high costs associated with SFE technologies. However, since the primary target product is generally found at trace concentrations, the residual algal biomass remaining in the vessel post-extraction represents a relatively unexplored and important secondary product potentially suitable as a highly marketable, protein-rich feed ingredient. As such, we investigated the potential nutritional value of whole *N. granulata* algal biomass and residual biomass after SFE processing at 70 and 90 °C in an initial exploratory study. Based solely on proximate, fatty acid and amino acid composition, these solvent-free *N. granulata* algal meals showed good potential for use in animal and fish feeds [6,7]. This finding is in agreement with recent work focused on the use of other *Nannochloropsis* species as feed inputs for aquaculture diets based on their attractive protein and amino acid profile and their ability to produce *n*-3 polyunsaturated fatty acids [8]. However, these studies did not report digestibility and further investigations with *Nannochloropsis* algal meals are required because the simple presence of high quality nutrients in novel feed ingredients does not ensure nutrient supply to target animal species. Specifically, the protein digestibility of *N. granulata* algal meals has not been previously reported

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for any aquaculture species. Additionally, good nutritional value in one target animal species does not necessarily guarantee the same in others due to differences in feeding habits and digestive physiologies such as those found between fish and shrimp (*e.g.*, slower gut transit time in trout, lack of an acidic stomach in shrimp). While the evaluation of protein quality *in vivo* is time-consuming and costly, *in vitro* assays that involve simulated digestion of test ingredient suspensions with 'species-specific' digestive enzymes can be highly informative with a minimal use of animal subjects, particularly once predictive regression equations have been developed. In addition, these methods are logistically attractive as they can be used to complement biochemical composition data, they are relatively inexpensive, results are rapidly obtained using small sample sizes, they side-step animal feed refusal issues until further *in vivo* tests can be performed and they are generally regarded as effective tools for making predictions of potential protein quality for research and industrial use prior to undertaking costly *in vivo* animal feeding trials. As such, the objective of this study was to predict the DP contents of three meals produced from a common lot of *N. granulata* biomass for Pacific white shrimp and rainbow trout using species-specific *in vitro* pH-Stat protein hydrolysis.

## 2. Materials and methods

### 2.1. Algal meals

The microalga species used in this study was the marine eustigmatophyte *Nannochloropsis granulata* Karlson and Potter (CCMP 535, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, East Boothbay, ME). This species was mass cultivated under continuous light in 1000 L Brite-Box™ photobioreactors. Details of mass cultivation, harvesting, processing and biochemical characterization of algal meals and lipid extracts have been previously described elsewhere [6,7]. General composition of the meals is shown in Table 1.

### 2.2. Shrimp and fish sampling

Hepatopancreas were sampled from six-hundred fed juvenile (7–10 g) Pacific white shrimp (*Litopenaeus vannamei*) reared in fertilized ponds on a commercial farm in the Northeast region of Brazil for crude enzyme extract. Shrimp cephalothorax was removed and hepatopancreas immediately excised, pooled into plastic vials on crushed ice (4 °C), rapidly frozen on dry ice and transported to the laboratory. The stomach and pyloric caeca of rainbow trout (*Oncorhynchus mykiss*) were sampled from ten healthy fed individuals (mean body weight 393.1 ± 35.8 g) farmed in freshwater raceways. Fish were killed by rapid cephalic concussion; digestive tract was excised, cleaned of visceral fatty tissue, and thoroughly cleansed with distilled water. The stomach and pyloric caeca were separated and pooled in plastic bags, frozen at −20 °C on site and transported to the laboratory frozen on dry ice.

**Table 1**
Proximate and caloric composition of *Nannochloropsis granulata* algal meals used for species-specific *in vitro* pH-Stat protein digestibility studies[a] (*as-fed* basis).

| | Base[b] | C70[c] | C90[c] | *P-value* |
|---|---|---|---|---|
| Moisture (%) | 3.4 ± 0.2[ns] | 4.0 ± 0.4 | 3.6 ± 0.5 | *0.058* |
| Ash (%) | 7.5 ± 0.0[a] | 8.3 ± 0.2[c] | 8.1 ± 0.1[b] | *<0.001* |
| Crude protein (%N × 4.78) | 33.9 ± 0.2[b] | 32.7 ± 0.6[a] | 33.7 ± 0.2[b] | *<0.001* |
| Crude lipid (%) | 27.6 ± 0.01[b] | 24.6 ± 1.2[a] | 24.7 ± 0.9[a] | *0.009* |
| Carbohydrate (%) | 14.4 ± 0.2[a] | 15.9 ± 0.6[b] | 15.0 ± 0.5[a] | *0.006* |
| Gross energy (MJ $kg^{-1}$) | 22.6 ± 0.0[b] | 22.0 ± 0.2[a] | 22.2 ± 0.1[a] | *0.002* |

ns = not significant ($P > 0.05$).
[a] Values within the same row having different superscript letters are significantly different ($P < 0.05$).
[b] Freeze-dried whole un-extracted *N. granulata* algal meal.
[c] Residual *N. granulata* algal meals after SFE processing at 35 MPa pressure for 270 min at 70 or 90 °C.

### 2.3. Recovery and standardization of crude digestive enzyme extracts

Shrimp digestive enzyme extracts were recovered after homogenization (T25 digital ultra-turrax®, 18G dispersing element, IKA WORKS, Inc., Wilmington, NC, USA) of pooled hepatopancreas with autoclaved chilled seawater (35 ppt salinity) (1:3 w/v), followed by centrifugation at 10,000 × *g* for 30 min at 4 °C. After elimination of the upper lipid layer, the supernatant was collected and pH of the enzyme extracts were adjusted to 8.0 with 0.1 N NaOH. Rainbow trout pyloric caeca samples were processed similarly but with distilled water (1:1 w/v). The stomach samples were also processed in distilled water (1:3 *w*/v) but the pH of the recovered enzyme extracts were adjusted to 2.0 with 0.1 N HCl. Enzyme extracts were stored in 2.0 mL labeled cryogenic vials and frozen (−20 °C) until analysis. All crude enzyme extracts were standardized according to their hydrolytic capacity using the *in vitro* pH-Stat method of determination of degree of protein hydrolysis (DH) according to Lemos et al. [9] and Yasumaru and Lemos [10]. Briefly, the extract volumes tested were 50, 200, 600 and 1000 μL using casein (shrimp and pyloric caeca extract) and haemoglobin (stomach extract) as substrates, which resulted in a log equation y = bLn(x)-a.

### 2.4. Species-specific in vitro pH-Stat determination of protein degree of hydrolysis (DH)

Following digestive enzyme extract standardization, the *in vitro* DH of the *N. granulata* algal meals was determined. The protein hydrolysis assay with shrimp hepatopancreas enzyme extract was conducted similarly to the alkaline enzyme extract standardization procedure. *N. granulata* algal meals (80 mg of protein) were stirred in seawater and pH automatically adjusted to 8.0 with 0.1 N NaOH. Hydrolysis assay started with the addition of the hepatopancreas enzyme extract (381 μL) and was carried out for 60 min at 25 ± 0.2 °C. For rainbow trout assays, algal meal samples were pre-hydrolyzed in distilled water with stomach crude enzyme extract (250 μL, pH 2.0, 60 min at 25 ± 0.2 °C) before the hydrolysis with pyloric caeca enzyme extract (200 μL, pH 8.0, 60 min at 25 ± 0.2 °C). Total enzymatic hydrolysis of 60 min for shrimp and 150 min (60 min stomach hydrolysis; 30 min transition; 60 min pyloric caeca hydrolysis) for rainbow trout were considered sufficient to hydrolyze proteins as references suggest that gut transit time for shrimp may vary from 60 to 90 min [12] and for rainbow trout it can be longer than 5 h at 9 °C [13] but with a rate increase of 2.1 times for each 10 °C increase in temperature [14]. The transition stage (30 min) between the hydrolysis with stomach and pyloric caeca extracts of rainbow trout consisted of pH adjustment and stabilization from pH 2.0 to 8.0 by the addition of 0.1 N NaOH. During the assays, nitrogen gas was purged into the reaction mixture. All DH assays were run in triplicate and protein ADC was predicted using published species-specific prediction equations [9,10]. Digestible protein (DP) content of the meals was calculated as follows: (DP = CP × [protein ADC / 100]).

### 2.5. Statistical methods

Data is reported as the mean ± standard deviation (n = 3). Statistical analyses were performed using one-way analysis of variance, ANOVA (SigmaStat® v.3.5) with a 5% level of probability ($P < 0.05$) selected in advance to sufficiently demonstrate a statistically significant difference. Where significant differences were observed, treatment means were differentiated using pairwise multiple comparison procedures (Tukey multiple range test). All raw data was confirmed to have a normal distribution using the Kolmogorov-Smirnov test (SigmaStat® v.3.5).

## 3. Results

The *N. granulata* meals used in the present study had highly similar proximate compositions with regard to moisture (3–4%), ash (7–8%),