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). Base freeze-dried N. granulata meal and meals processed using supercritical fluid extraction (SFE) with CO2 at 70 and 90°C had highly similar nutritional compositions with regard to their contents of moisture (3-4%), ash (7-8%), crude protein (N×4.78; 33-34%), crude lipid (25-28%), total carbohydrate (14-16%) and gross energy (22-23 MJ kg-1). Predominant essential amino acids included leucine (3.1-3.2%), arginine (2.5%), lysine (2.0-2.3%) and valine (2.1-2.2%). 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 3.56% (P=0.351) and predicted ADC of 83.4% (P=0.366). Alternatively, meals processed 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 5.05% and predicted ADC of 88.0%, compared to 3.23% and 81.6%, respectively. Predicted protein ADC was high (>82%) for all N. granulata meals for both Pacific white shrimp (83-84%) and rainbow trout (82-88%) and therefore indicates very good potential for use in fish and shrimp diets. Based on our results, we suggest DP values for similar N. granulata meals of 28% (as-fed basis) or 30% (dry matter basis) for juvenile Pacific white shrimp and rainbow trout.

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J.A. Olivares, PhD
Editor-in-Chief, Algal Research

Dear Dr. Olivares,

On behalf of my co-authors, please find enclosed our manuscript entitled "Prediction of digestible protein contents of marine microalgae (*Nannochloropsis granulata*) meals for Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*) using 'species-specific' *in vitro* pH-Stat protein hydrolysis". We confirm that this work has not been nor will be submitted elsewhere during its review process and we thank-you for your consideration of its publication in Algal Research.

All the best,

Sean M. Tibbetts, PhD

Prediction of digestible protein contents of marine microalgae (*Nannochloropsis granulata*) meals for Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*) using 'species-specific' *in vitro* pH-Stat protein hydrolysis.

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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*). Base freeze-dried *N. granulata* meal and meals processed using supercritical fluid extraction (SFE) with CO_2 at 70 and 90°C had highly similar nutritional compositions with regard to their contents of moisture (3-4%), ash (7-8%), crude protein (N×4.78; 33-34%), crude lipid (25-28%), total carbohydrate (14-16%) and gross energy (22-23 MJ kg^{-1}). Predominant essential amino acids included leucine (3.1-3.2%), arginine (2.5%), lysine (2.0-2.3%) and valine (2.1-2.2%). 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 3.56% (P=0.351) and predicted ADC of 83.4% (P=0.366). Alternatively, meals processed 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 5.05% and predicted ADC of 88.0%, compared to 3.23% and 81.6%, respectively. Predicted protein ADC was high (>82%) for all *N. granulata* meals for both Pacific white shrimp (83-84%) and rainbow trout (82-88%) and therefore indicates very good potential for use in fish and shrimp diets. Based on our results, we suggest DP values for similar *N. granulata* meals of 28% (*as-fed* basis) or 30% (dry matter basis) for juvenile Pacific white shrimp and rainbow trout.

Keywords: Microalgae; *Nannochloropsis*; Protein digestibility, Degree of hydrolysis, Fish, Shrimp

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 more than 50% of all seafood consumed globally today and this proportion is projected to rise to 62% by 2030 [1,2]. With a strong push towards economic and ecological sustainability, this sector requires additional alternatives to conventional feed inputs [3]. 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 [4] 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 [5]. 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 [6-9]. 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 [10]. 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 [10,11]. 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 [12]. 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 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 palatability issues 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 [13]. Details of mass cultivation, harvesting, processing and biochemical characterization of algal meals and lipid extracts have been previously described elsewhere [10,11]. To generate the meals tested in this study, freeze-dried biomass (Base) was pulverized to a fine powder (to pass through a 500 µm screen) using a laboratory ultra-centrifugal mill (Retsch model ZM200, Retsch GmbH., Haan, Germany). Portions (350 g each) of the Base were then subjected to supercritical CO_2 fluid extraction (SFE) using a pilot-scale SFE instrument (DynaSep LLC, Newark, DE, USA) at constant CO_2 pressure (35 MPa), flow

101 rate (100 g min^{-1}) and residence time (270 min.) at 70 or 90°C (C70 and C90). Proximate and caloric
102 contents and essential amino acid concentrations are presented in Table 1 for reference.
103
104 2.2. Shrimp and fish sampling
105 Hepatopancreas were sampled from six-hundred juvenile (7-10 g) Pacific white shrimp (*Litopenaeus*
106 *vannamei*) reared in fertilized ponds on a commercial farm in the Northeast region of Brazil for crude
107 enzyme extract. Shrimp cephalothorax was removed and hepatopancreas immediately excised, pooled into
108 plastic vials on crushed ice (4°C), rapidly frozen on dry ice and transported to the laboratory. The stomach
109 and pyloric caeca of rainbow trout (*Oncorhynchus mykiss*) were sampled from ten healthy individuals
110 (mean body weight 393.1±35.8 g) farmed in freshwater raceways. Fish were killed by rapid cephalic
111 concussion; digestive tract was excised, cleaned of visceral fatty tissue, and thoroughly cleansed with
112 distilled water. The stomach and pyloric caeca were separated and pooled in plastic bags, frozen at -20°C
113 on site and transported to the laboratory frozen on dry ice.
114
115 2.3. Recovery of crude digestive enzyme extracts
116 Shrimp digestive enzyme extract was recovered after homogenization (T25 digital ultra-turrax®, 18G
117 dispersing element, IKA WORKS, Inc., Wilmington, NC, USA) of pooled hepatopancreas with autoclaved
118 chilled seawater (35 ppt salinity) (1:3 w/v), followed by centrifugation at 10,000 × *g* for 30 min at 4°C.
119 After elimination of the upper lipid layer, the supernatant was collected and pH of the enzyme extract was
120 adjusted to 8.0 with 0.1 N NaOH. Rainbow trout pyloric caeca sample was processed similarly but with
121 distilled water (1:1 w/v). The stomach sample was also processed in distilled water (1:3 w/v) but the
122 recovered enzyme extract pH was adjusted to 2.0 with 0.1 N HCl. Enzyme extracts were stored in 2.0 mL
123 labeled cryogenic vials and frozen (-20°C) until analysis.
124
125 2.4. Standardization of crude digestive enzyme extracts
126 Crude enzyme extracts were standardized according to their hydrolytic capacity using the *in vitro* pH-
127 Stat method of determination of degree of protein hydrolysis (DH). Briefly, in the pH-Stat concept, the
128 cleavage of peptide bonds by the enzyme extract results in a pH shift (increase or decrease depending on

129 acid or alkaline hydrolysis, respectively), which is automatically stabilized by the addition of a titrant. The
130 volume of titrant added is proportional to the DH by the digestive enzyme extract. For example, at a
131 constant pH of 8.0, the amount of titrant consumed is proportional to the amount of peptide bonds cleaved
132 [14,15]. Standard protein substrates for stomach (acid) and pyloric caeca or hepatopancreas (alkaline)
133 assays were analytical grade hemoglobin from bovine blood (95% crude protein, H2625, Sigma-Aldrich,
134 St. Louis, MO, USA) and casein from bovine milk (90% crude protein, C7078, Sigma-Aldrich, St. Louis,
135 MO, USA), respectively. Standardization consisted of determination of the hydrolytic capacity of different
136 enzyme extract volumes from stomach, pyloric caeca or hepatopancreas over the same substrate amount
137 (80 mg of protein). Assays were carried out simultaneously in two automated titrators with double
138 measuring interfaces with burettes (Titrando 836, Titrando 907 - Metrohm AG, Switzerland), connected to
139 a single controlling and data logging software (Tiamo™ v. 2.2, Metrohm AG, Switzerland). Standard
140 substrate samples (80 mg of protein) were stirred in distilled water or seawater in the reaction vessel (8.0
141 mL total suspension volume) and the pH automatically adjusted to 2.0 for the acid or 8.0 for the alkaline
142 assays by the addition of 0.1 N HCl or 0.1 N NaOH, respectively, and kept stable for 30 (hemoglobin) and
143 60 (casein) minutes. The suspension final volume was adjusted to 10 mL (including the enzyme extract)
144 and protein hydrolysis assays were carried out for 60 minutes. The reaction temperature was maintained at
145 25±0.2°C in jacketed reaction vessels connected to a heated/refrigerated constant-temperature water bath
146 (temperature uniformity ±0.1°C, RSWB 3222A Lindberg/BlueM, Thermo Electron Corp., MA, USA).
147 During the assays, nitrogen gas was purged in the mixture and the vessel covered with plastic film to avoid
148 interference of atmospheric CO_2 in the reaction pH.

149 The DH was calculated according to Adler-Nissen [14] as:

150 $$DH = B \times Nb \times (1/\alpha) \times (1/MP) \times (1/H_{tot}) \times 100\%$$

151 Where:

152 B = volume of titrant consumed (mL)

153 Nb = normality of the titrant

154 α = average degree of dissociation of the α-NH groups ($1/\alpha$=1.50 for pH 8.0 at 25°C)

155 MP = mass of substrate protein (g)

156 H_{tot} = total number of peptide bonds in the protein substrate (7.6-9.2 meqv g protein^{-1}, according to the
157 source of protein assayed) [14]
158
159 2.5. Species-specific *in vitro* pH-Stat determination of protein degree of hydrolysis (DH)
160 Following digestive enzyme extract standardization, the *in vitro* DH of the *N. granulata* algal meals
161 was determined. The protein hydrolysis assay with shrimp hepatopancreas enzyme extract was conducted
162 similarly to the alkaline enzyme extract standardization procedure. *N. granulata* algal meals (80 mg of
163 protein) were stirred in seawater and pH automatically adjusted to 8.0 with 0.1 N NaOH. Hydrolysis assay
164 started with the addition of the hepatopancreas enzyme extract and was carried out for 60 min at 25±0.2°C.
165 For rainbow trout assays, algal meal samples were pre-hydrolyzed with stomach crude enzyme extract (pH
166 2.0, 60 minutes at 25±0.2°C) before the hydrolysis with pyloric caeca enzyme extract (pH 8.0, 60 minutes
167 at 25±0.2°C). During the assays, nitrogen gas was purged into the reaction mixture. All DH assays were run
168 in triplicate and protein ADC was predicted using published species-specific prediction equations [16,17].
169
170 2.6. Statistical methods
171 Data is reported as the mean±standard deviation (n=3). Statistical analyses were performed using one-
172 way analysis of variance, ANOVA (SigmaStat® v.3.5) with a 5% level of probability ($P<0.05$) selected in
173 advance to sufficiently demonstrate a statistically significant difference. Where significant differences were
174 observed, treatment means were differentiated using pairwise multiple comparison procedures (Tukey
175 multiple range test). All raw data was confirmed to have a normal distribution using the Kolmogorov-
176 Smirnov test (SigmaStat® v.3.5).
177
178
179 **3. Results**
180 Proximate and caloric contents of *N. granulata* algal meals (*as-fed* basis) are shown in Table 1. All
181 meals had highly similar nutritional compositions with regard to their contents of moisture (3-4%), ash (7-
182 8%), crude protein (N×4.78; 33-34%), crude lipid (25-28%), total carbohydrate (14-16%) and gross energy
183 (22-23 MJ kg^{-1}). Although significant differences ($P\leq0.009$) were observed, it is related to the low variance

between analytical replicates and likely of little biological significance. Essential amino acid compositions of *N. granulata* algal meals (*as-fed* basis) are shown in Table 2. With the except of lysine, no significant differences (P≥0.308) were found for any essential amino acids between the three algal meals with pooled average contents of 2.5% (arginine), 0.7% (histidine), 1.7% (isoleucine), 3.1% (leucine), 0.8% (methionine), 1.8% (phenylalanine), 1.7% (threonine), 0.03% (tryptophan) and 2.1% (valine). The content of lysine for the temperature-treated meals (2.1%) was significantly lower (P=0.019), and statistically similar to each other (P=0.587), than for the untreated base meal (2.3%). Species-specific *in vitro* pH-Stat protein DH, predicted protein ADC and DP contents of *N. granulata* algal meals are shown in Table 3. Protein DH and predicted ADC values of all *N. granulata* meals were statistically similar (P≥0.351) for Pacific white shrimp with averages of 3.6% (range, 3.41-3.70%) and 83% (range, 82.9-83.9%), respectively. The resultant DP content of *N. granulata* meals for Pacific white shrimp was 28% (*as-fed* basis) or 29% (dry matter basis). For rainbow trout, the DH of temperature-treated meals (5.0%) were significantly higher (P<0.001), and statistically similar to each other (P=0.191), than for the untreated base meal (3.2%). The same was true for predicted protein ADC (P<0.001) with averages of 88 and 82%, respectively. The resultant DP contents of *N. granulata* meals for rainbow trout were positively correlated (r=0.99; P<0.001) with increasing processing temperature as follows: 28% (Base) < 29% (C70) < 30% (C90) on an *as-fed* basis or 29% (Base) < 32% (C70) < 33% (C90) on a dry matter basis.

4. Discussion

There has been growing interest in recent years in *Nannochloropsis* microalgae as potential feedstocks for various industrial applications. The major focus has been on *N. oculata* and *N. gaditana* related to their potential supply of fat-soluble vitamins and carotenoids [18,19], n-3 essential fatty acids [20-23], bioactive compounds [24-26] and their suitability for biofuels production [27-29] with some limited focus on their protein fraction [30-32]. By comparison, *N. granulata* has received far less attention; focused mainly on its lipid accumulation potential for biodiesel production [11] and glycoproteins as bioactive compounds [33,34]. With regard to the nutritional quality of the biomass that can be produced from *N. granulata* for food/feed applications, only a scant amount of data is available. Initial work in our lab with whole-cell and

212 lipid-extracted biomass demonstrated that they may have some potential as a protein-rich animal and/or
213 aquafeed ingredient based on their relatively high crude protein contents of up to 44% (N×4.78) [35] or
214 54% (N×6.25), comparatively low ash content for a marine microalgae (<8%), richness in essential amino
215 acids like leucine (>9 g 100 g^{-1} protein) and lysine (>6 g 100 g^{-1} protein), high essential amino acid indices
216 (>0.9), low total phenolic content (<8 mg GAE g^{-1}) and moderately high (>84%) *in vitro* indirect pH-Drop
217 protein digestibility using a multi-enzyme cocktail consisting of porcine-derived pancreatic trypsin and
218 intestinal peptidase and bovine-derived pancreatic α-chymotrypsin [10,36]. However, much of this work
219 was preliminary and comparative in nature and further investigations involving species-specific *in vitro*
220 protein quality and, ultimately, *in vivo* biological performance of target animals fed diets supplemented
221 with these products are critical next steps.

222 Beyond general proximate composition, the essential amino acid profile and protein digestibility are
223 generally the most important criteria that define the nutritional value of feed ingredients. Regarding the
224 essential amino acid profiles of the *N. granulata* meals used in this study, the concentrations of most (9 of
225 10) amino acids were unaffected by temperature treatment relative to the freeze-dried base meal.
226 Temperature treatment did cause a modest, but significant, reduction in lysine content for the temperature-
227 treated meals relative to the untreated base meal and was linearly related to increasing processing
228 temperature in a previous study [10]. Lysine is typically the first essential amino acids affected by thermal
229 processing of feedstuffs [37] and is related to the free amino group on the epsilon carbon unit of lysine that
230 is highly susceptible to reactions with reducing sugars under exposure to elevated temperatures [38]. Given
231 that lysine was only reduced from 2.3 to 2.1% and all other essential amino acid levels were statistically
232 similar between *N. granulata* meals, it is likely that the protein quality will be largely dependent upon its
233 digestibility. As for protein digestibility of conventional feed ingredients, this is highly dependent on their
234 solubility and ultimate potential for chemical hydrolysis and enzymatic digestion in the digestive tract;
235 which is often influenced by post-harvest production conditions such as exposure to high temperatures
236 processing [39,40] and its effect on amino acid profile and protein quality as previously discussed.
237 However, for novel ingredients derived from single-cell proteins, like microalgae, the recalcitrant cell wall
238 is another major factor that can limit nutrient digestibility in the gut of monogastric animals, including fish
239 and shrimp [41]. As a general rule, it is felt that the upper dietary inclusion limit of algae co-products in

feeds for most commercially-relevant farmed terrestrial monogastric animals is about 15% and the cause has been attributed to low digestibility associated with these rigid algal cell walls; but also because of reduced feed intakes associated with poor palatability of algae-supplemented diets [42-45]. However, none of the microalgae species involved were from the genus *Nannochloropsis* and, it is our opinion, that the recommendations are largely based on studies that failed to actually measure the digestibility of the tested algal products prior to animal feeding trials. While both of these limitations can be somewhat overcome through advanced feed processing and rational diet formulation, difficult-to-rupture cellulosic algal cell walls are a legitimate concern for most microalgae-based feed ingredients. Specifically, all species in the genus *Nannochloropsis* are encased in a rigid cell wall made up primarily of an inner cellulose layer surrounded by an outer layer of algaenan [46]. This outer algaenan coating may make the cells relatively hydrophobic, which could reduce the immediate solubility upon entering the monogastric stomach. Additionally, since the digestive tract of monogastric animals essentially lack any appreciable cellulase enzyme activity, rupture of these cells walls either prior to inclusion in the feed or by the highly acidic gastric juices in the monogastric stomach after consumption is requisite to efficient utilization of the intracellular nutrients supplied by *Nannochloropsis*-based ingredients. As such, some workers have made attempts to determine the nutritional value of *Nannochloropsis*-based ingredients for food/feed applications with varying results. As for farmed marine aquaculture species, recent studies conducted with red drum, *Sciaenops ocellatus*, gilthead seabream, *Sparus aurata* and European seabass, *D. labrax* have demonstrated that dietary supplementation with various microalgae, including a related *Nannochloropsis* species (*N. salina*), up to 25% is acceptable based on growth performance, nutrient utilization, carcass yields, organ weights, sensory evaluation, digestive enzyme activities and intestinal histological parameters [47-49] which 'suggests' good digestibility in these species. In stark contrast, Skrede et al. [50] used mink as a 'model' monogastric species for aquaculture and indirectly estimated the protein digestibility of a related *Nannochloropsis* species (*N. oceanica*) to be very low (35%). To our knowledge, the only study to report the *in vitro* protein digestibility of *N. granulata* algal biomass is that of Tibbetts et al. [36] who found it to be relatively high for whole-cell (84-85%) and lipid-extracted (88-91%) meals. While this study used an indirect *in vitro* pH-Drop method which makes the results preliminary, comparative in nature and, most importantly, not species-specific, the moderately high predicted protein digestibilities (>84%) found were

encouraging and warranted a further examination of protein digestibility of similar *N. granulata* algal meals using *in vitro* protein digestibility methods that are more representative of real-scenario digestibility using species-specific digestive enzymes such as those used in the present study. In fact, the results obtained in this study agree well with the previous preliminary results having found highly similar predicted protein ADC values for Pacific white shrimp (83-84%) and rainbow trout (82-88%).

While the DH and predicted protein ADC values found in the present study are similar to important practical aquafeed ingredients; such as fish meals [16], there may be room for further improvement of the present algal meals for fish and shrimp nutrition. Although the temperature treatment of the algal meals produced increased hydrolytic capacity of trout digestive enzymes, it was not enough to produce significant differences, compared to the untreated sample, for shrimp. An improved treatment regime; possibly combining higher processing temperature, pressure and residence time, may result in a more complete liberation of intracellular constituents and ultimately higher algal protein digestion in shrimp as evidenced for other terrestrial plant-based ingredients and aquatic single-cell proteins and seaweeds [51-54]; similar to values observed for trout in this study. Relative to finfish such as trout, shrimp exhibit relatively rapid food transit times through the gut [55] so a high reliability on digestive enzyme efficiency is paramount for ingested nutrient recovery in their relatively simple digestive tract [56]. The observed protein DH values with shrimp enzymes suggest a reasonably high digestive capacity for the untreated base material (3.4%); which even exceeded those with trout enzymes (3.2%). On the other hand, the mild heat treatment proved highly successful for improving protein digestion with fish enzymes (4.9-5.2%) by pre-hydrolysis with a gastric acidic phase followed by digestion with pyloric caeca alkali crude enzyme extracts. As such, it is probable that the lack of a gastric phase in the shrimp DH assay is related to the marginal, but non-significant, improvement in protein digestion of algal meals subjected to a mild heat treatment (3.6-3.7%). The present DH, predicted protein ADC and DP values should provide useful species-specific data for use and further improvement of *N. granulata* algal meals as potential sustainable ingredients for aquafeeds.

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Conflict of interest

The authors declare no conflict of interest.

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519 Table 1

520 Proximate and caloric composition of *Nannochloropsis granulata* algal meals used for species-specific *in*

521 *vitro* pH-Stat protein digestibility studies[a] (*as-fed* basis).

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

529 [a] Values within the same row having different superscript letters are significantly different (P<0.05).

530 [b] Freeze-dried whole un-extracted *N. granulata* algal meal.

531 [c] Residual *N. granulata* algal meals after SFE processing at 35 MPa pressure for 270 min. at 70 or 90°C.

532

Table 2

Essential amino acid 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*
Arginine	2.46±0.02[ns]	2.50±0.12	2.54±0.12	*0.740*
Histidine	0.72±0.00[ns]	0.75±0.05	0.74±0.04	*0.814*
Isoleucine	1.68±0.01[ns]	1.69±0.02	1.73±0.04	*0.308*
Leucine	3.13±0.01[ns]	3.12±0.01	3.19±0.08	*0.409*
Lysine	2.33±0.01[b]	2.10±0.02[a]	2.05±0.08[a]	*0.019*
Methionine	0.84±0.01[ns]	0.84±0.02	0.87±0.04	*0.542*
Phenylalanine	1.85±0.00[ns]	1.85±0.03	1.86±0.02	*0.777*
Threonine	1.59±0.00[ns]	1.72±0.14	1.74±0.08	*0.353*
Tryptophan	0.03±0.00[ns]	0.03±0.00	0.04±0.01	*0.480*
Valine	2.08±0.02[ns]	2.13±0.05	2.19±0.11	*0.422*

[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.

Table 3

Species-specific *in vitro* pH-Stat degree of hydrolysis (DH), predicted apparent digestibility coefficients (ADC) for protein and digestible protein (DP) content of *Nannochloropsis granulata* algal meals[a] (n=3).

	Base[b]	C70[c]	C90[c]	*P-value*
Pacific white shrimp				
DH (%)	3.41±0.13[ns]	3.70±0.30	3.58±0.21	*0.351*
Predicted ADC[d] (%)	82.9±0.5[ns]	83.9±1.0	83.5±0.7	*0.366*
DP (%, as-fed basis)	28.1±0.2[ab]	27.5±0.3[a]	28.2±0.2[b]	*0.025*
DP (%, dry matter basis)	29.1±0.2[ns]	28.6±0.3	29.2±0.2	*0.055*
Rainbow trout				
DH (%)	3.23±0.12[a]	4.93±0.09[b]	5.17±0.20[b]	*<0.001*
Predicted ADC[e] (%)	81.6±0.4[a]	87.6±0.3[b]	88.4±0.7[b]	*<0.001*
DP (%, as-fed basis)	27.6±0.1[a]	28.7±0.1[b]	29.8±0.2[c]	*<0.001*
DP (%, dry matter basis)	28.8±0.1[a]	31.9±0.1[b]	32.6±0.3[c]	*<0.001*

[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.

[d] Prediction equation: ADC = ([-0.1033 + 139.88DH] ÷ [1 + 1.355DH + 0.011DH^2]) according to Lemos et al. [16].

[e] Prediction equation: ADC = (3.5093DH + 70.248) according to Yasumaru and Lemos [17].

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