Algal Research 11 (2015) 359–367

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel

David W. Templeton, Lieve M.L. Laurens *

National Bioenergy Center, National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA

ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 3 June 2015
Accepted 20 July 2015
Available online 12 August 2015

Keywords:
Nitrogen-to-protein conversion factor
Biofuel
Bioproduct
Amino acid
Protein
Total nitrogen
Non-protein nitrogen

ABSTRACT

Accurately determining protein content is important in the valorization of algal biomass in food, feed and fuel markets. Conversion of elemental nitrogen to protein is a well-accepted and widely practiced method, but depends on developing an applicable nitrogen-to-protein conversion factor. The most complete method to determine this factor takes six different hydrolyses of the subject material and these are not always carried out in reported literature studies. We report new data for conservative conversion factors determined from 21 algae samples along with over 50 amino acid profiles from the literature, representing distinct cultivation conditions for fresh and marine algae. We find that the amino acid profile among different algae samples is consistent, however the large variability between strains in non-protein nitrogen (up to 54% in microalgae) causes variability in the calculated conversion factor. We include our calculated novel nitrogen-to-protein conversion factors for model and commercially relevant biofuel algal strains and compare these with the literature.

1. Introduction

The protein content of algae has strong influence in determining potential food, feed, co-product and fuel uses for algal biomass [1,2]. For example, in the context of an algal biofuel production pathway, the value of the residual biomass (after oil removal) depends on its compositional characteristics and affects the overall process economics [3–5]. Research to develop economically viable algal biofuel or bioproduct pathways is urgently needed and future deployment of successful processes will depend on reducing production costs and finding value from all biomass components [6–10]. As part of the overall technoeconomic modeling and process optimization there is a need to accurately track algal biomass components in and out of different unit operations and this includes accurate quantification of the protein content [11,12]. Good component mass balance accounting gives added confidence that each of the components has been accurately measured. The protein content of microalgae can range from 7% to 40% [1,2,13,14] and can change dramatically over the course of its lifecycle [15]. One source of biomass that is currently commercialized for food supplement sale thanks to the high protein content (reported to be >50–60% of the biomass) is *Spirulina* [16]. Thanks to this high protein content the biomass can contribute to human diet supplementation [17]. Protein content determination in *Spirulina* in commercial preparations is often based on the common determination using a 6.25 factor [18] and a reassessment of the protein versus non-protein nitrogen determination in mass cultivated biomass is needed but has not been carried out for this organism [16,19].

In order to develop viable algal bioproduct processes and to assess multiple process conditions, protein analysis methods need to balance analyte specificity, precision and accuracy with method robustness, ease of use and low cost. Analytical methods to determine protein, for food labeling purposes, have been reviewed by Moore et al. [20] and include 1) copper or dye binding spectroscopic techniques, 2) UV or IR techniques, 3) amino acid (AA) analysis hydrolysis methods, and 4) elemental nitrogen analysis which is converted to protein using a nitrogen-to-protein conversion factor.

The use of spectrophotometric methods can be useful for generating relative protein data, but can be less useful for determining absolute protein values, which are needed for component balance calculations. In the case of the Lowry spectrophotometric procedure, the color development is based on the reduction of the Folin reagent (Cu^{2+} to Cu^{+}) by aromatic residues and peptide bonds in protein, after which the Cu^{+} is chelated by bicinchoninic acid (BCA) to form the detected color [21–23]. Because the Folin reagent will react with other reducing substances in solution, this assay is susceptible to algae species- and growth condition-specific interferences which often cause a high bias [15,24]. All spectrophotometric methods depend on complete extraction of all proteins from the biomass matrix, but it is difficult to completely solubilize all the cytosolic, structural, membrane bound or other protein types found in algae in order to expose them to the colorimetric reagent external to the cell. In addition, the choice of a standard protein for calibration

* Corresponding author.
E-mail address: Lieve.Laurens@nrel.gov (L.M.L. Laurens).

http://dx.doi.org/10.1016/j.algal.2015.07.013
2211-9264/

is critical, since its response must be similar to that of the released sample proteins. This poses an additional uncertainty in that a typical standard protein may or may not represent the average protein composition found in algal strains. A large discrepancy between the Lowry protein assay and a nitrogen-to-protein factor-based calculation was observed for three strains harvested at different stages of nutrient deprivation and shown to be highly dependent on the physiological and biochemical status of the cells [15].

The most direct method for protein determination is by acid hydrolysis (often a 6M HCl digestion for 24 h) followed by HPLC amino acid analysis [25]. This method has the advantage of breaking down the biological matrix and does not depend on selective removal of protein from the biomass. Free amino acids can also be detected in the hydrolysate along with AAs hydrolyzed from proteins. However, for complete AA analysis multiple (up to six) hydrolyses for each sample are needed to completely quantify the chemically diverse amino acids found in proteins [26]. In addition to the typical 24 h HCl hydrolysis, separate hydrolyses are needed for Trp and for the sulfur containing AAs (Met and Cys). Two additional hydrolysis timepoints (12 h and 48 h) are run to account for AAs (Thr, Ser and Tyr) that are partially degraded during hydrolysis plus a separate 2 h ammonia hydrolysis is run to determine the NH_3 released from Gln and Asn. Direct amino acid quantification after several hydrolyses per sample is a useful method, it is also expensive and time consuming and thus less applicable for screening or processing a large number of samples. A combined method can harness the completeness and specificity of the direct AA analysis with a simpler, higher throughput nitrogen analysis method (%N) by using an appropriate nitrogen-to-protein conversion factor.

Calculating protein using a nitrogen-to-protein conversion factor is not subject to spectral interferences or protein extraction efficiency differences since the entire sample is consumed during the %N analysis. The %N analytical methods, either combustion (Dumas method) or Kjeldahl, are simple, fast and inexpensive compared to hydrolysis followed by AA analysis. They can be run on multiple samples and can be easily adapted to process monitoring or timepoint analyses. The %N methods have the disadvantage of not being specific for protein nitrogen but rather they measure the *total* nitrogen found in the sample. Algae have many nitrogen containing components such as chlorophyll, nucleic acids (DNA/RNA) and amino sugars (e.g. glucosamine, galactosamine) in addition to protein. This non-protein nitrogen (NPN) needs to be properly accounted for within the nitrogen-to-protein conversion factor. In their determinations of algal protein conversion factors Lourenço et al. [13,14] quantitated the major NPN classes (chlorophyll, nucleic acids and inorganic N) in algae samples and the NPN accounted for about 15–30% (with some above 40%) of the total nitrogen in the algae. These authors were able to close the nitrogen balance to around 90–95% including the protein nitrogen.

Jones [27] described differences in nitrogen content of food and how nitrogen-to-protein factors would need to be adjusted for different foods. Tkachuk [28,29] determined factors for wheat plus cereals and oilseeds. Several cereals were analyzed for nitrogen and amino acid content by Mossé and colleagues [30,31]. Mossé suggests a method to determine a useful nitrogen-to-protein conversion factor [26] for use in food and feed nutritional analysis, and describes how to determine upper (k_A) and lower (k_P) limits for this factor and ultimately suggests combining these into a single, averaged factor (k). These factors can be easily defined mathematically, as in Eqs. (1) and (2), though there are many practical, analytical and computational pitfalls to avoid when calculating these factors.

$$k_A = (\sum E_i)/(\sum D_i) \quad (1)$$

$$k_P = \sum E_i/N. \quad (2)$$

The term $\sum E_i$ is the sum of the amino acid residues or the anhydrous amino acids (AAA), accounting for the mass loss during polymerization into proteins. The term $\sum D_i$ is the sum of the nitrogen content of each of the AA residues including ammonia released during hydrolysis. The term N refers to the %N found in the samples by combustion or Kjeldahl methods and includes both protein and non-protein nitrogen (NPN) found in the sample.

The first factor, k_A, is calculated by determining the sum of anhydrous amino acids (AAA) divided by the sum of the %N found within these AAAs. However, the k_A factor assumes all nitrogen measured comes from protein (i.e. NPN = 0) and this is true only for purified protein samples. For biomass samples, k_A will over-predict protein values due to the presence of NPN [26]. The second factor, k_P, is estimated by the sum of AAA divided by the total %N, which includes any NPN found in the sample. For biomass samples using k_P to calculate protein assumes the NPN content is similar as in the calibration samples. As a practical matter k_A is an upper bound to the conversion factor and k_P is a lower bound. Mossé makes the argument that the best conversion factor (k) for protein in real samples is an average of k_A and k_P.

The key to using %N as a predictor of protein content is to have access to a useful nitrogen-to-protein conversion factor for the samples being analyzed. A common method to determine crude protein utilizes the historical conversion factor of 6.25 times the %N value. This factor, which Yamaguchi [32] traces back to the year 1839, tends to overestimate protein in most biomass and even food applications and has been criticized by several authors [33–36]. For food and feed applications, specific nitrogen-to-protein conversion factors have been previously reported. Diniz et al. [37] determined nitrogen-to-protein conversion factors (k_P) of 5.39 to 5.98 for nine species of fish from Brazilian coastal waters. Sriperm et al. [36] calculated all three different types of conversion factors for various feedstuffs and determined k_A values of 5.68 for corn, 5.64 for soybean meal, 5.74 for corn dry distillers grain (DDGS), 5.45 for poultry by-product meal and 5.37 for meat and bone meal. Nitrogen-to-protein conversion factors for microalgae have been reported recently and an overall average k_i factor of 4.78 was reported and is often used [13,14,38,39]. The general trend for reported and specifically calculated factor appears to be much lower than the traditional 6.25 factor. Other authors have previously mentioned the difficulties in evaluating a conversion factor and related this to additional evidence that the cell wall of algae plays an important role in protein quantification [40].

Upon thorough review of the literature on food, feed, and algae applications of nitrogen-to-protein conversion factors, there appear to be inconsistencies between factors (k_A, k_P or k) reported for calculating protein content measurements. In addition to a review and literature data mining study, we report new data for all three k factors analyzed from 21 algae samples, representing distinct cultivation stages and fresh and marine microalgal strains. This allows us to compare the amino acid profile of microalgae between strains and investigate the origins of the nitrogen-to-protein conversion factors. We recalculate k factors from literature reports, where primary data are also reported, and revise them to be on a consistent and comparable basis. We compare the effects of including different analytical tests on the calculations for the k factors. We report on differences in literature reported k values and make recommendations on the best approaches to produce and utilize the k factors for protein determination in algal bioprocess research.

2. Materials and methods

2.1. Sample selection

A total of 21 algal biomass samples were selected to represent a range of different types of algae that are relevant to ongoing outdoor cultivation and biomass production scenarios. Cultivation conditions have been described before [15]. In brief, biomass from three strains, *Scenedesmus* sp. (LRB-AP 0401), *Chlorella* sp. (LRB-AZ 1201) and *Nannochloropsis* sp. was provided by Arizona State University and

represents harvests taken in early-, mid-, and late-cultivation stages or high-protein (greater than 30% DW protein), high-carbohydrate (greater than 30% DW total biomass carbohydrates), and high-lipid (greater than 30% DW total lipid) content biomass, respectively. By timing the harvest, biomass of different composition was obtained in a controlled fashion in outdoor flat panel (650 L) photobioreactors in nitrate deplete cultivation media. Cultivation time after reaching nutrient deplete conditions depended on final target biomass composition desired, which, depending on the growing season, was 3 to 5 days for high carbohydrate (midpoint harvest) biomass and 6 to 9 days for high lipid (late harvest) biomass. High protein (early harvest) biomass was obtained by harvesting prior to nutrient depletion. In addition to the controlled cultivation samples, we included the following samples that were obtained through collaborations or were grown at NREL: *Neochloris oleoabundans* (#5033), *Phaeodactylum tricornutum* (#4742 and 5016), *Chlorella vulgaris* UTEX 395 (#4739), *Nannochloropsis salina* (NS1-Solix, #4743), *Nannochloropsis* sp. (Seambiotic, #5031) and *Nanofrustulum* sp. (#5018).

2.2. Amino acid determination

Lyophilized algal biomass was analyzed for AAs according to AOAC reference method 994.12 [25] by a commercial laboratory in St. Paul, MN (AminoAcids.com). Three of the amino acids, Trp, Met and Cys, are destroyed during the standard 24 h 6M HCl hydrolysis, so additional hydrolysis tests included a performic acid oxidation hydrolysis for the sulfur-containing amino acids (Met and Cys), and a separate alkaline hydrolysis for Trp. Free amino acids in algae can make up a significant (3–12%) fraction of the algal dry weight [41]. In our work, these AAs are measured in addition to the AAs liberated during hydrolysis and are counted as protein-amino acid and included in our factor calculations.

2.3. Elemental nitrogen determination

Elemental nitrogen content (on a dry weight basis) was determined by a commercial laboratory (Huffman Laboratories, Golden, CO), using a combustion (Dumas) method.

2.4. Calculation of factors

Table 1 shows the underlying data and conversion factors needed to translate the amino acid data plus %N data into nitrogen-to-protein conversion factors, including the average molecular mass and the mass the amino acid residue and the percentage of nitrogen found in both the amino acid and the corresponding residue. The nitrogen content per AAA varies from about 9% (Tyr) to 36% (Arg) (Table 1) and thus knowledge of the composition of all AAs is crucial for the accurate quantification of a useful conversion factor. Amino acid values are presented on many bases in the literature (g AA per 100 g protein; mg AA per g of sample N; g AA per 16 g N), though for algae process analysis and comparisons, reporting AAs on a dry weight basis is most convenient. Otherwise, reporting all data needed to convert to a dry weight basis would be helpful.

A spreadsheet was developed to calculate the different nitrogen to protein conversion factors for NREL grown microalgae. The AA concentrations were converted to amino acid residues (anhydrous amino acids, AAA) to account for the loss of water during the polymerization of the AAs during hydrolysis using the data found in Table 1. This spreadsheet utilizes AA data (on a gram dry algal biomass basis), converts the data to an amino acid residue basis and calculates the amount of nitrogen found in each residue.

For comparison purposes, nitrogen to protein conversion factors from literature references were also re-calculated using the same spreadsheet (included as Supplemental Table 1). Where necessary, the original data was converted to a g AA per dry weight basis to facilitate the calculations. Differences of <0.05 units between the revised calculation and the original reported value are thought to be insignificant and explained by rounding errors. Occasional calculation errors were discovered in the reported literature values and the revised (and corrected) conversion factors are reported in our Supplemental Table 1 where it was possible to reproduce the original reported literature value. Non-protein AAs such as hydroxyproline, taurine, lanthionine, hydroxylysine and ornithine, which can be co-reported with AA data, are not included in this calculation, and data reported on a % protein basis were normalized to exclude these components. Factors were calculated using different combinations of initial data in order to assess the effect of different measuring options.

Table 1
Basic values used to calculate nitrogen-to-protein conversion factors (k_A, k_P, k); AA = amino acid, AAA = anhydrous amino acid.

Amino acid	Abbreviation	Formula residue	Avg. mass (Da)	Mass residue (Da)	Ratio AAA/AA	%N in AA	%N in AAA
Aspartic acid	Asp	$C_4H_5NO_3$	133.1038	115.0886	0.865	10.5%	12.2%
Threonine	Thr	$C_4H_7NO_2$	119.1203	101.1051	0.849	11.8%	13.9%
Serine	Ser	$C_3H_5NO_2$	105.0934	87.0782	0.829	13.3%	16.1%
Glutamic acid	Glu	$C_5H_7NO_3$	147.1307	129.1155	0.878	9.5%	10.8%
Proline	Pro	C_5H_7NO	115.1319	97.1167	0.844	12.2%	14.4%
Glycine	Gly	C_2H_3NO	75.0671	57.0519	0.760	18.7%	24.6%
Alanine	Ala	C_3H_5NO	89.0940	71.0788	0.798	15.7%	19.7%
Cysteine	Cys	C_3H_5NOS	121.1540	103.1388	0.851	11.6%	13.6%
Valine	Val	C_5H_9NO	117.1478	99.1326	0.846	12.0%	14.1%
Methionine	Met	C_5H_9NOS	149.2078	131.1926	0.879	9.4%	10.7%
Isoleucine	Ile	$C_6H_{11}NO$	131.1746	113.1594	0.863	10.7%	12.4%
Leucine	Leu	$C_6H_{11}NO$	131.1746	113.1594	0.863	10.7%	12.4%
Tyrosine	Tyr	$C_9H_9NO_2$	181.1912	163.1760	0.901	7.7%	8.6%
Phenylalanine	Phe	C_9H_9NO	165.1918	147.1766	0.891	8.5%	9.5%
Tryptophan	Trp	$C_{11}H_{10}N_2O$	204.2284	186.2132	0.912	13.7%	15.0%
Lysine	Lys	$C_6H_{12}N_2O$	146.1893	128.1741	0.877	19.2%	21.9%
Histidine	His	$C_6H_7N_3O$	155.1563	137.1411	0.884	27.1%	30.6%
Arginine	Arg	$C_6H_{12}N_4O$	174.2027	156.1875	0.897	32.2%	35.9%
Asparagine	Asn	$C_4H_6N_2O_2$	132.1190	114.1038	0.864	21.2%	24.6%
Glutamine	Gln	$C_5H_8N_2O_2$	146.1459	128.1307	0.877	19.2%	21.9%
Ammonia	–	NH_3	17.031	16.023	0.941	82.2%	87.4%
Water	–	H_2O	18.01524	–	–	–	–
Mass hydrogen	–	H	1.008	–	–	–	–
Mass nitrogen	–	N	14.007	–	–	–	–

3. Results and discussion

3.1. Recalculated literature k values

In order to study the effect of different *k* factor calculations on protein content predictions, we mined literature data for conversion factor information and calculations with the aim of filling gaps in the literature where all three factors were not originally reported and compare the different permutations of AA tests used to calculate the factors. We have calculated *k* factors for a novel data set of algal samples harvested from controlled cultivation experiments for select strains. Our data set includes a range of materials, including fresh and marine organisms as well as representative biomass for early and late harvest samples. These lifecycle biomass samples were selected to allow us to look at the dependence of the calculated conversion factor on the biological origin of the biomass.

Using the spreadsheet described in the methods section, we recalculated all three different *k* factors (k_A, k_P and *k*) from literature reports that included amino acid, NH_3 and %N data. Summary information about these reports is listed in Table 2, which shows the different combinations of hydrolysis conditions used in the published calculations. According to Mossé [26], the most complete method to determine the total amino acid content takes 6 different hydrolyses along with NH_3 analysis, though this is rarely found in literature reports. Only 3 out of the 15 reports, shown in Table 2, calculate the *k* factors following all 6 recommended hydrolyses. Most researchers use fewer analyses and we include a discussion of the effect of missing different hydrolyses. The k_A and k_P factors from the 3 literature reports that contain all 6 recommended hydrolyses are plotted in Fig. 1A, and represent food and feed sources, ranging from corn grains, soy, to cheese, milk and beef and fish samples (based on data shown in Supplemental Table 1). This figure also includes trends of k_A and k_P calculated as if less than all 6 hydrolyses were run and shows the effect missing hydrolyses have on calculating the three *k* factors. The upper two traces of Fig. 1A show the k_A factor without the NH_3 result while the next two traces show the same k_A data including the NH_3 result. The inclusion of the NH_3 test has a large effect on the k_A factor calculation showing a difference of around 0.76 units. This is due to the effect of NH_3 testing on the k_A denominator as it strongly affects the sum of the nitrogen found within the amino acid residues. Conversely the presence or absence of the NH_3 test does not affect the k_P calculation. The bottom two (actually 4 overlapping) traces on Fig. 1A show this. Thus the NH_3 test affects the k_A, and therefore the *k* factor, but not k_P factor and the most correct method to determine the k_A and *k* would include a separate NH_3 hydrolysis even though this is rarely reported in the literature.

Fig. 1A and B also shows the effect of including the special hydrolysis data for the acid-labile amino acids Met, Cys and Trp on calculating the factors. The upper 4 traces show that the k_A factor calculated from 18 AAs is slightly higher than the comparable 15 AA k_A factor (calculated without acid labile AA data). The effect of 18 vs 15 AAs is more pronounced for the k_P factor than the k_A factor. We found for the new microalgal samples that the effect of the special hydrolyses on k_A averaged to 0.07 units, 0.22 units for k_P and 0.15 units for *k*.

Only 2 of the 15 reports included multiple hydrolysis timepoints to account for minor losses of labile AAs (especially Thr, Ser and Tyr) seen during HCl hydrolysis. A calculation with and without the timepoint hydrolyses showed a negligible effect with differences of less than 0.05 units seen. Including multiple timepoint hydrolyses is more accurate and can be included for completeness, though skipping these does not affect the *k* factor values much and comes with large cost and time savings. Some researchers report results for Cys, Met or Trp based on the normal 24 h HCl hydrolysis condition, which severely degrades these AAs. When compared to results from the normal 15AA adding results from these three AAs (derived from suboptimal hydrolysis conditions) does not significantly change the calculated *k* factors (data not shown). Thus, reporting Cys, Met or Trp based on the normal 24 h HCl hydrolysis (rather than special hydrolysis conditions) does not help and special hydrolysis conditions are needed to accurately determine these AA concentrations. Morr showed the effect of calculating the *k* factors using hydrous amino acid data versus anhydrous amino acid residue data [42,43]. The results were first reported on a hydrous AA basis then later recalculated on the more correct AAA basis. The "hydrated" values were seen to be about 0.9–1.0 units higher than the correctly calculated anhydrous basis, so calculating these values using AA residue data is critical.

3.2. Common basis k factor comparison

Fig. 1B illustrates k_A and k_P factors calculated on literature macroalgae or seaweeds values (samples 1–27), literature microalgal values (28–50), and new microalgae results (51–71) reported here. All these factors are calculated based on a common basis of a reduced set of 15 AA analyses without NH_3 to capture the largest number of algae comparisons and are not comparable to *k* factors calculated from the complete 6 hydrolysis data presented in Fig. 1A. The k_A values are similar among the three data sets (averaging 6.2) while the k_P factors

Table 2
Table of literature reported *k* factors and the comparison AA hydrolysis conditions used to calculate the factors.

Amino acid hydrolysis conditions						Factors reported			Protein matrix	Ref.
24 h AA hydrolysis	Multiple timepoints	Separate Cys & Met tests[a]	Separate Trp test[b]	NH_3[c]	Cys, Met or Trp[d]	k_A	k_P	*k*		
x	x	x	x	x		x	x	x	Corn grain	[31]
x		x	x	x		x	x	x	Food products	[36]
x		x	x	x		x	x		Soy	[43]
x		x	x			x			Food products	[47]
x	x	x	x			x	x	x	Microalgae	this work
x		x	x				x		Microalgae and extracts	[48]
x		x	x			x	x		Microalgae and extracts	[49]
x				x	x		x		Microalgae	[13]
x				x	x				Seaweed (macroalgae)	[39]
x				x	x		x		Microalgae	[14]
x				x	x	x	x	x	Seaweed (macroalgae)	[50]
x						x	x	x	Microalgae	this work
x									Microalgae	[51]
x							x		Seaweed (macroalgae)	[52]
x							x		Fish	[37]

[a] Analyzed from performic acid oxidized and hydrolyzed material.
[b] Analyzed from alkaline hydrolysis in $Ba(OH)_2$.
[c] Analyzed from separate 2 h acid hydrolysis.
[d] Analyzed using suboptimal acid hydrolysis conditions.

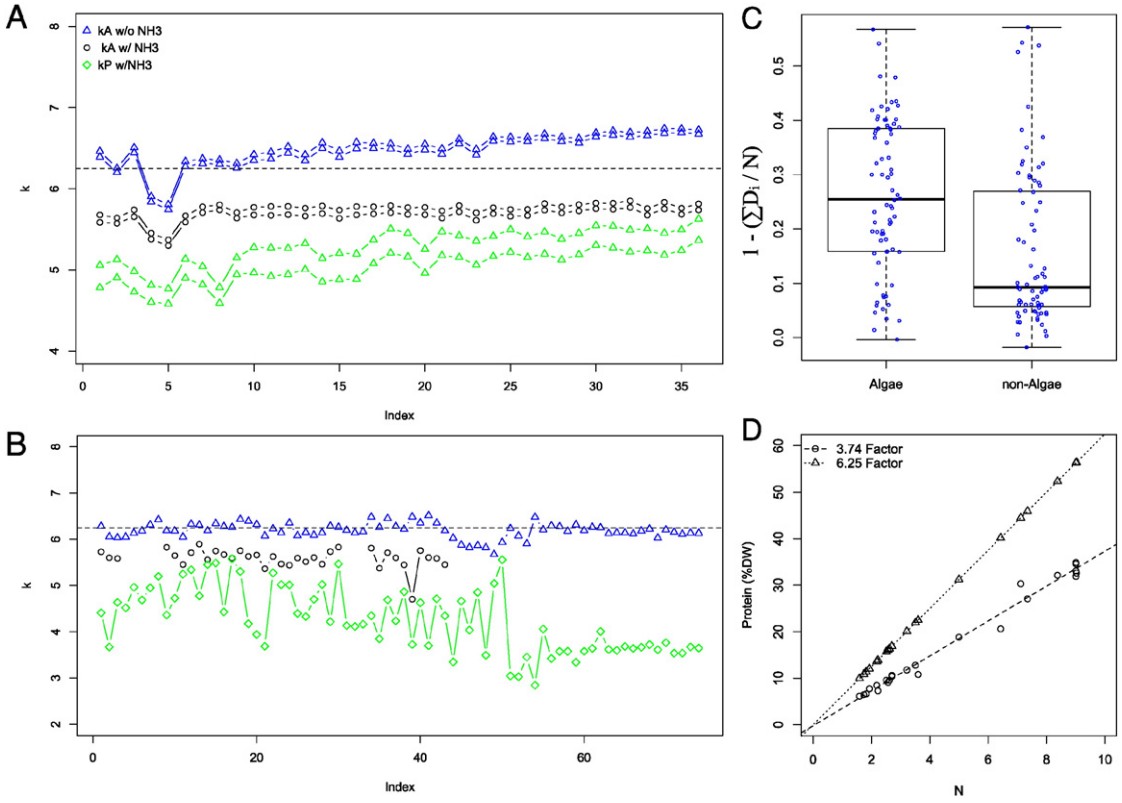

Fig. 1. Influence of amino acid data and non-protein-nitrogen (NPN) content on k_A and k_P factors and protein content calculated. (A) Literature-derived factors for *k* for a variety of food and feed ingredients, ranging from corn and soy to cheese, milk, beef and fish (data indices listed in supplemental raw data file). The upper two traces (blue triangles) show k_A calculated without NH_3 data while next two traces show k_A calculated including NH_3 data (black circles). The smaller difference seen in these pairs of traces show the effect of including special hydrolyses for Cys, Met and Trp. The bottom two traces (actually 4 traces, green diamonds) show no effect on NH_3 testing on k_P and some effect of the Cys, Met, and Trp special hydrolyses. A horizontal dashed line showing the traditional 6.25 conversion factor is included as a reference, (B) comparison of *k* factors for all macro- and micro-algae from literature set and NREL data set (macroalgae or seaweeds values (indices 1–27), literature microalgal values (28–50), and new microalgae results (51–71) reported here), symbols and color schemes identical to (A). Data are calculated on a 15AA basis without NH_3 measurements. (C) Data distribution presented as a Tukey boxplot of calculated NPN content in algae and non-algae samples, the median value of the data sets is shown as a solid horizontal black line, the interquartile range (IQR) is shown as a box around the median value, with the 'whiskers' indicating the values that fall within 1.5 IQR. (D) Conversion of nitrogen to protein content with two different conversion factors and correlation with anhydrous AA residue calculations (shown as protein content % DW), circles represent the relationship between elemental nitrogen content and protein, calculated 3.74 as the slope of the linear correlation between %N and the sum of anhydrous amino acids (Ei). Triangles represent the protein content, calculated based on the traditional factor of 6.25.

are 4.75, 4.40 and 3.55 for macroalgae, literature microalgae and our work, respectively. A single factor ANOVA on the three data groups (macro algae, literature microalgae and new microalgae data) showed the average k_A values are similar (p-value 0.52) while the k_P factors were statistically significantly different (p-value 2×10^{-12}). The cause of the similarity in the k_A factors lie in the similarities of the amino acid profiles among the samples, whereas the k_P difference could lie in the measurement of the %N value. Since the $\sum E_i$ value is common to both k_A and k_P calculation and the $\sum D_i$ used to calculate k_A is derived from the same AA data used to determine k_A, changes to the %N value must be the cause of the differences seen in the k_P factors. This suggests that the %N measurement could be subject to interferences or the growth conditions for microalgae were very different. Differences in %N seen in these samples could also be due to different amounts of NPN in algae, different amounts of interfering nitrogen from the growth media (incomplete washing of biomass), differences in the analytical techniques used to determine %N, or differences in the algal species chosen for analyses by the different groups.

We calculated the NPN fraction, (as $1 - \sum D_i$,) in the algal samples (both micro- and macroalgae) and found that the NPN averaged 26.2%. While for the non-algae samples in the data set (including corn, fish, meats and other food products) the NPN averaged 15.9%, which is significantly different ($p < 0.05$) as seen in Fig. 1C. This NPN calculation by difference depends on using the most complete (six hydrolyses) analysis of amino acids to ensure that any unmeasured protein is not counted with NPN. It has been reported that the best estimate of the conversion factor is the average of k_A and k_P, [26] although the information obtained from this published work on edible seeds with low nitrogen levels (2–6% dry weight) and low NPN levels (~5%) may not be transferable to algae. In particular, because the algae samples analyzed here have much higher range of nitrogen (1.59–9.01%) and higher levels of NPN (up to 54%, Table 3 and Fig. 1C). This suggests that the k_P value may be a better choice for algal samples. However, it is likely that different algal strains will require a dedicated analysis and conversion factor calculation. This is supported by earlier reports highlighting the lack of a universal conversion factor for all species [40].

3.3. k factor choices

The choice of conversion factor can have a large effect on the amount of protein estimated in an algal sample (Fig. 1D). Here we have determined the k_A (6.24) k_P (3.71) and *k* (4.97) for the 21 new algal samples shown in Table 3. On average, for all microalgal samples combined between literature and new data, the k_P factor is 4.08, however, the large variability shown in Fig. 1B for k_P indicates that each strain may require their own k_P conversion factor calculation. Based on the existing literature and the assumption that the non-protein nitrogen portion of algal biomass may vary, we found a linear correlation between the nitrogen content and the sum of anhydrous amino acids. The slope (3.74), according to the Mossé definition (Eq. (1) above) reflects the

Table 3
AA profile of 21 different algal biomass samples without NH_3 analysis. Also includes derived values needed to calculate k_A, k_P and k nitrogen-to-protein conversion factors. ND = not determined

Weight % AA	Based on 18AA and no NH_3 measurement														Based on 15 AA and no NH_3 measurement						
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
L-aspartic acid	3.64	0.70	0.65	3.85	1.13	1.15	0.93	2.92	0.83	2.77	1.31	1.39	3.23	1.38	1.56	0.96	1.40	0.74	3.63	2.20	1.08
L-threonine	2.13	0.50	0.45	1.88	0.58	0.59	0.49	1.64	0.50	1.15	0.63	0.74	1.79	0.60	0.91	0.58	0.69	0.38	1.82	1.18	0.61
L-serine	1.67	0.36	0.34	1.65	0.50	0.51	0.41	1.35	0.39	1.02	0.56	0.59	1.49	0.53	0.68	0.43	0.58	0.31	1.57	1.01	0.50
L-glutamic acid	4.22	0.74	0.73	4.98	1.33	1.38	1.04	3.94	1.00	3.03	1.43	1.52	4.18	1.30	1.80	1.11	1.68	1.13	4.98	2.83	1.22
L-proline	1.87	0.44	0.39	1.93	0.59	0.61	0.48	1.51	0.43	1.56	0.48	0.63	2.87	0.47	0.84	0.50	0.73	0.40	1.98	1.50	0.67
L-glycine	2.05	0.42	0.39	2.18	0.66	0.67	0.52	1.65	0.47	1.12	0.55	0.77	1.97	0.61	0.86	0.53	0.82	0.43	2.13	1.23	0.61
L-alanine	3.12	0.69	0.67	3.45	1.23	1.26	1.07	2.70	0.75	1.56	0.70	0.94	2.50	0.74	1.39	0.85	1.25	0.71	3.19	1.66	0.84
L-cysteine[a]	0.66	0.20	0.18	0.52	0.19	0.19	0.18	0.52	0.22	0.34	0.24	0.12	0.29	0.23	ND	ND	ND	ND	ND	ND	NR
L-valine	2.33	0.52	0.46	2.34	0.73	0.75	0.62	1.74	0.56	1.32	0.69	0.90	2.27	0.67	1.04	0.63	0.90	0.50	2.40	1.39	0.71
L-methionine[a]	0.93	0.24	0.19	0.90	0.29	0.30	0.23	0.66	0.23	0.55	0.28	0.29	0.67	0.26	ND	ND	ND	ND	ND	ND	ND
L-isoleucine	1.63	0.36	0.32	1.64	0.49	0.50	0.40	1.17	0.38	1.14	0.54	0.66	1.72	0.57	0.73	0.42	0.62	0.32	1.60	1.04	0.51
L-leucine	3.43	0.75	0.65	3.73	1.15	1.17	0.92	2.55	0.73	1.84	0.84	1.24	3.33	0.93	1.51	0.87	1.43	0.74	3.67	2.16	1.04
L-tyrosine	1.47	0.28	0.26	1.72	0.51	0.52	0.41	1.47	0.32	0.87	0.43	0.52	1.48	0.48	0.60	0.36	0.63	0.33	1.60	0.95	0.48
L-phenylalanine	2.17	0.49	0.42	2.48	0.73	0.74	0.59	1.54	0.44	1.36	0.61	0.86	1.96	0.66	0.97	0.55	0.92	0.45	2.17	1.34	0.65
L-tryptophan[b]	0.84	0.17	0.15	0.84	0.25	0.27	0.17	0.72	0.15	0.38	0.14	0.22	0.62	0.19	ND	ND	ND	ND	ND	ND	ND
L-lysine	2.33	0.38	0.39	2.64	0.74	0.75	0.60	1.86	0.50	1.51	0.89	0.37	2.00	0.67	0.92	0.56	0.88	0.49	3.65	1.54	0.76
L-histidine	0.67	0.09	0.10	0.81	0.24	0.24	0.18	0.67	0.13	0.42	0.18	0.23	0.75	0.19	0.25	0.15	0.31	0.16	0.89	0.49	0.23
L-arginine	2.34	0.40	0.43	2.79	0.76	0.77	0.63	2.94	0.48	2.02	0.63	0.61	2.24	0.66	0.90	0.54	0.91	0.48	2.78	1.44	0.72
Total AA	37.49	7.73	7.19	40.32	12.10	12.40	9.88	31.52	8.51	23.97	11.12	12.61	35.35	11.14	14.95	9.05	13.75	7.57	38.06	21.96	10.62
Total AAA or $\sum$Ei	32.13	6.61	6.15	34.59	10.36	10.62	8.45	27.07	7.29	20.60	9.55	10.80	30.31	9.56	12.78	7.73	11.77	6.48	32.62	18.81	9.09
$\sum D_i$	5.13	1.03	0.98	5.58	1.67	1.71	1.36	4.52	1.15	3.35	1.50	1.65	4.84	1.50	2.04	1.24	1.90	1.04	5.41	3.03	1.48
%N	8.38	1.82	1.59	9.01	2.70	2.70	2.18	7.35	2.22	6.43	2.51	3.60	6.84	2.62	3.51	1.93	3.21	1.74	9.03	5.00	2.57
Non-protein N (%)	38.8	43.4	38.4	38.1	38.1	36.7	37.6	38.5	48.2	47.9	40.2	54.2	29.2	42.7	41.9	35.8	40.8	40.2	40.1	39.4	42.4
k_A	6.26	6.41	6.29	6.20	6.22	6.22	6.20	5.99	6.32	6.15	6.35	6.54	6.27	6.37	6.26	6.25	6.18	6.22	6.03	6.20	6.16
k_P	3.83	3.63	3.87	3.84	3.84	3.93	3.88	3.68	3.28	3.20	3.81	3.00	4.43	3.65	3.64	4.01	3.67	3.72	3.61	3.76	3.54
k	5.05	5.02	5.08	5.02	5.03	5.08	5.04	4.84	4.80	4.68	5.08	4.77	5.35	5.01	4.95	5.13	4.92	4.97	4.82	4.98	4.85

1—*Scenedesmus* sp. (early); 2—*Scenedesmus* sp.(mid); 3—*Scenedesmus* sp. (late);4—*Chlorella vulgaris* (early); 5–6—*C. vulgaris* (mid, technical duplicates); 7—*C. vulgaris* (late); 8—*C. vulgaris* UTEX395; 9—*Neochloris oleoabundans*; 10—*Phaeodactylum tricornutum* (source 1); 11—*P. tricornutum*(source 2); 12—*Nannochloropsis salina*; 13—*Nannochloropsis* sp. (source 3); 14—*Nannofrustulum*; 15—*Scenedesmus* sp.; 16–19—*C. vulgaris*; 20—*Nannochloropsis* sp. (source 4); 21—*Nannochloropsis* sp. (source 5).

[a] Analyzed after performic acid oxidation.

[b] Analyzed after alkaline hydrolysis.

k_P conversion factor. For contrast, protein data obtained with a 6.25 conversion factor is shown in Fig. 1D, which is often utilized to estimate protein values even though this was not originally developed for algae or plant samples. In this example the 6.25 conversion factor leads to an average of 40.8% overestimation of the protein content.

3.4. New algal AA data

The basis of our nitrogen-to-protein conversion factors can be found in the AA composition and %N content of the biomass for 21 microalgal biomass samples shown in Table 3. Total AA (sum of AA) content ranged from 7.17% to 40.32% dry weight, which corresponds to 6.15% to 34.59% dry weight on a total AAA basis. Samples 1–3 and 4–7 were sampled at three different lifecycle stages (early, mid, late respectively) corresponding to high protein, high carbohydrates and high lipid samples. While large changes in total protein amount are seen in these lifecycle samples, no trend in the k factors is seen. Samples 5 and 6 are technical replicates and are included to show the reproducibility of the AA measurements to within 0.02 wt% for most amino acids and to within 0.3% for total amino acids. The AA variability did not affect the k_A factor though a difference of 0.09 units for k_P and 0.05 units for k is seen. As with many literature reports (Table 2), these samples were not subjected to NH_3 hydrolysis. Samples 1–14 were analyzed with special tests for Cys, Met, and Trp (total 18 AA) while samples 15–21 were subjected to only the 24 h HCl hydrolysis (total 15 AA).

The AA profile visualized in Fig. 2A shows the relatively stable contribution of 18 individual AAs for a subset of 4 biomass samples, representing 3 distinct phylogenetic groups, but grown in consistent nutrient replete growth conditions (correlating with high biomass protein content). There is no clear trend that can be discerned among these species except that the most variability between the strains can be observed in the AAs: Asp, Glu, Ala, Val, Leu, Lys and Arg. This observation is consistent with early literature stating that no significant AA content trends could be found that related to the taxonomy of the organisms [44]. When we look at just one species (*Chlorella* sp.) and study its AA profile over the course of nutrient depletion (in this case nitrate deprivation), we notice that only a select set of AA are affected by the metabolic stress condition of the cells; Glu, Ala, and minor contributions from Lys and Arg (Fig. 2B). The other AAs showed consistent AA content through this algal lifecycle. The observations can be attributed to changes in the metabolic rearrangements of functional proteins upon nutrient deprivation and in amino acid catabolism [45]. The reduction observed in Glu is likely due to the joint action of enzymes in glutamate synthetase and synthase, both implicated in the main route of nitrogen assimilation or remobilization into amino acids needed to sustain the metabolic needs of the cell [46]. The other amino acids changes observed can be linked with direct changes in amino acid metabolism, which along with carbohydrate active enzymes are highly represented in the increasing and decreasing groups of proteins differentially regulated upon nutrient stress [45].

Because the nitrogen-to-protein conversion factors are calculated using the sum of the AA residues, if the relative composition and profile of the AA varies, the factor might be affected. However, we calculated the factors for a range of different algal samples and noticed no significant effect on the factor that we can attribute to the cultivation or phylogenetic information of the samples, suggesting that k factor changes balance each other. When we looked at the calculated factors

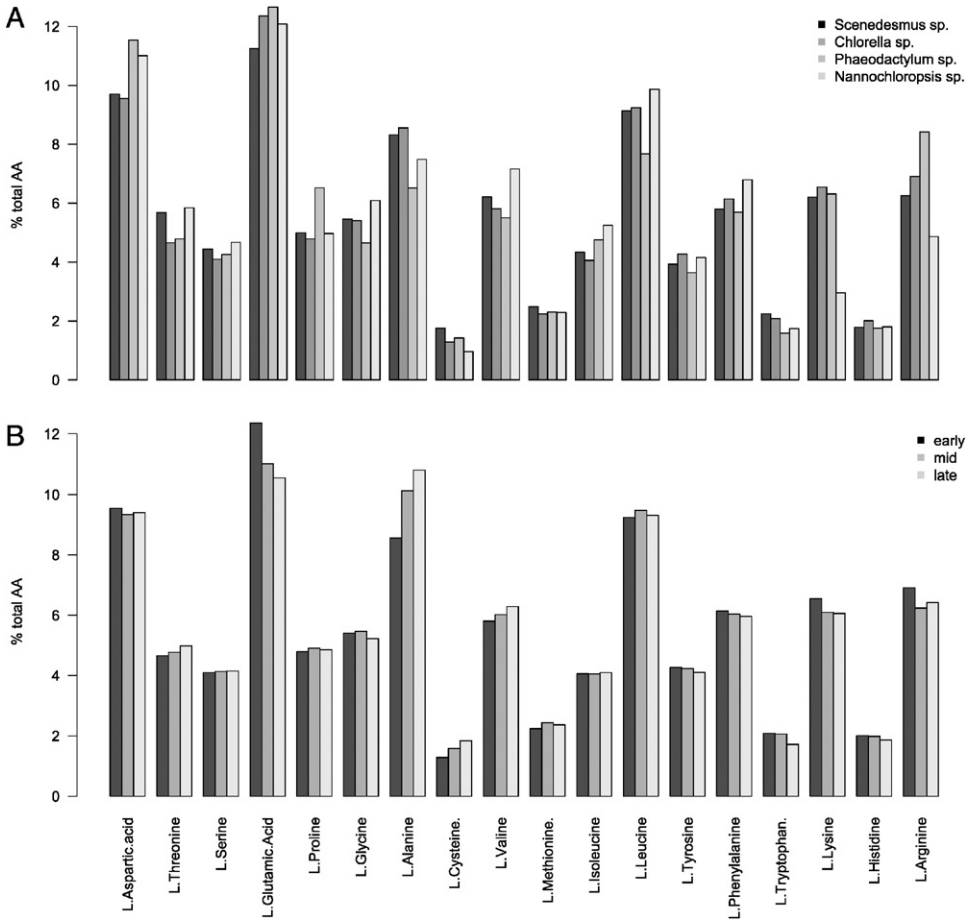

Fig. 2. Amino acid profile diversity; (A) typical amino acid profile for *Scenedesmus* sp., *Chlorella* sp., *Phaeodactylum* sp. and *Nannochloropsis* sp.; (B) Dynamic amino acid profile of *Chlorella* sp. over the course of nutrient depletion. All data are shown as % contribution to total amino acid content.

(Table 3) we did not see a trend that corresponds with timing of harvest. This is in apparent contradiction with previous literature for microalgae indicating variability of conversion factor with changing physiological condition (i.e. nitrogen limitation) [13,14]. Based on this published information, the late harvest factors would be significantly higher than the early harvests, which is not the case here.

As an additional way to look for an influence of the amino acid profile on the distribution between the different organisms, we used a principal component analysis. The more distinct the amino acid profiles are, the more likely this would be reflected in a grouping on a PCA scores plot (Fig. 3A). Specifically, when we compared the amino acid profiles between all the algae samples we have analyzed, representing fresh and marine organisms and various growth rates, we could detect a grouping of marine and fresh water organisms, though the influence is minor (up to 21% of all variability in the dataset). Upon further investigation of the contribution of the individual amino acids to the groupings (loadings plots for PC1 and PC2, Fig. 3B–C, respectively), we noticed that the major amino acids that contribute to this distinction are Asp and Pro for PC1 and Glu, Pro, Phe, and Arg for PC2 When we compared our data to previously published work, we notice a distinction between datasets that is primarily driven by Glu and Asp (data not shown), which, as the high nitrogen containing amino acids, can partially account for the small differences in calculated factors relative to the literature.

There are no significant trends in calculated conversion factor that explain a correlation between the three main classifications of algae; green algae, diatoms and Eustigmatophyceae (*Nannochloropsis* sp.) or between the different cultivation scenarios that were used to generate the biomass (early or late stage cultivation). The AA analysis indicates that the composition is relatively well distributed between all 18 AAs measured and consistent between the 21 different samples. Even though these analyses did not include NH_3, the impact of those measurements would be mostly on the accurate respective quantification of Gln versus Glu and Asn versus Asp. Since the molecular weight of Gln and Glu, and Asn and Asp are within 1 Da of each other, the lack of NH_3 measurements would therefore not affect the factor that is solely dependent on the amino acid content and composition (k_P), however, the contribution of nitrogen to the amino acids in the distribution (Di) would change and therefore significantly impact the k_A factor calculations.

Quantitatively determining the total amount of protein in an algal biomass matrix is difficult despite the multitude of analytical techniques available. For the analysis of protein in microalgal biomass samples for biofuel process development the use of a nitrogen to protein conversion factor combined with total protein analysis is a good combination of accuracy and speed. Despite the popularity of this method, three different forms of the conversion factor are reported along with different degrees of completeness. Factor calculations are related to the amino acid composition and the non-protein nitrogen content of the biomass. We demonstrate that in the absence of accurate quantification of the non-protein nitrogen content, and ammonia data, the best factor to use is k_P, which, as the sum of amino acids making up the polymeric protein structure, most closely reflects the actual protein content of the biomass. The k_A factor is a poor choice for conversion factor since it assumes all nitrogen comes from protein, or NPN equals zero. This factor overestimates the true protein content of a sample such as algal biomass and can be strongly biased high due to the presence of NPN in the biomass and the inclusion of NH_3 in the data, which is necessary for accurate calculation of this factor. The k factor may be the most accurate choice but it depends on both k_A and k_P factors and requires the most

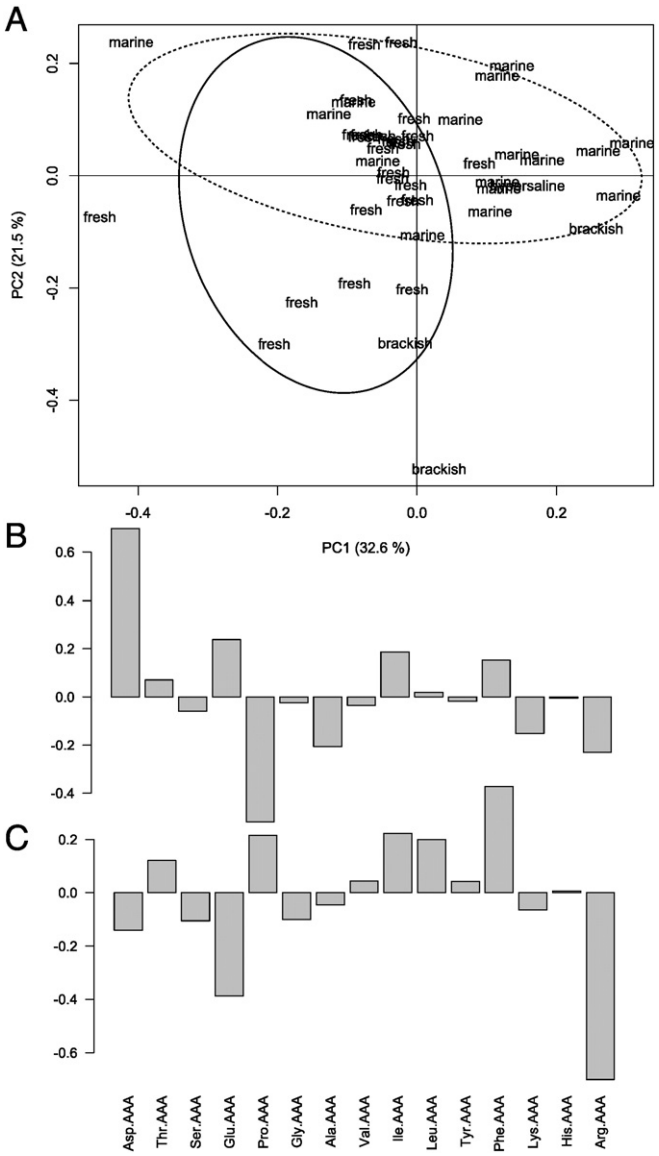

Fig. 3. Principal component analysis (PCA) scores for PC1 vs PC2 (A) and loadings for PC1 and PC2 respectively (B—C) plots of the amino acid profile of a total of 46 samples analyzed for acid stable amino acids, subdivided in groups of algae in their environment, fresh, brackish, marine, hypersaline water, positioning of marine and fresh water species are indicated with dashed and solid line respectively.

extra tests. The k_p factor is the most conservative choice, but it more properly takes into account the NPN in the sample, assigns all amino acids to protein and is the easiest practical analysis. From the published literature and new data we have analyzed, it is likely that algae require a dedicated factor and future reporting will need to state which factor is used for measurements, as well as a justification and a matching of a factor with groups of organisms.

In our work we calculated a conservative factor for the strains and biomass samples characterized in detail. However a more conservative factor used in protein characterization in algal biomass can have significant commercial implications. The final recommendation from this work for algal biomass producers, researchers and trade organizations is to highlight the requirement for a dedicated, often highly strain- and process-specific conversion factor. Even with a conservative choice of *k* factor, microalgal biomass often contains much more protein than terrestrial plant material and similar amounts to animal meats (30–50%). The k_P factor is a relatively quick and easy though conservative method to account for only protein defined as the sum of amino acids in algal biomass.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2015.07.013.

Acknowledgments

We acknowledge Chris Scarlata, Nicholas Sweeney, Stefanie Van Wychen at NREL for valuable discussions and sample preparation. This work was supported by the U.S. Department of Energy under Contract No. DE-AC36-08-GO28308 with the National Renewable Energy as part of the BioEnergy Technology Office (BETO), under task 1.3.4.300.

References

[1] O. Pignolet, S. Jubeau, C. Vaca-Garcia, P. Michaud, Highly valuable microalgae: biochemical and topological aspects, J. Ind. Microbiol. Biotechnol. 40 (2013) 781–796.
[2] E.W. Becker, Micro-algae as a source of protein, Biotechnol. Adv. 25 (2007) 207–210.
[3] R. Davis, A. Aden, P.T. Pienkos, Techno-economic analysis of autotrophic microalgae for fuel production, Appl. Energy 88 (2011) 3524–3531.
[4] R.H. Wijffels, M.J. Barbosa, An outlook on microalgal biofuels, Science 329 (2010) 796–799.
[5] P.J.L.B. Williams, L.M.L. Laurens, Microalgae as biodiesel & biomass feedstocks: review & analysis of the biochemistry, energetics & economics, Energy Environ. Sci. 3 (2010) 554–590.
[6] L. Brennan, P. Owende, Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products, Renew. Sust. Energ. Rev. 14 (2010) 557–577.
[7] E. Suali, R. Sarbatly, Conversion of microalgae to biofuel, Renew. Sust. Energ. Rev. 16 (2012) 4316–4342.
[8] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: a review, Renew. Sust. Energ. Rev. 14 (2010) 217–232.
[9] A. Bahadar, M.B. Khan, Progress in energy from microalgae: a review, Renew. Sust. Energ. Rev. 27 (2013) 128–148.
[10] J.R. Ziolkowska, L. Simon, Recent developments and prospects for algae-based fuels in the US, Renew. Sust. Energ. Rev. 29 (2014) 847–853.
[11] J. Benemann, Microalgae for biofuels and animal feeds, Energies 6 (2013) 5869–5886.
[12] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96.
[13] S.O. Lourenço, E. Barbarino, U.M.L. Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors, J. Phycol. 34 (1998) 798–811.
[14] S.O. Lourenço, E. Barbarino, P.L. Lavín, U.M. Lanfer Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors, Eur. J. Phycol. 39 (2004) 17–32.
[15] L.M.L. Laurens, S. Van Wychen, J.P. McAllister, S. Arrowsmith, T.A. Dempster, J. McGowen, et al., Strain, biochemistry, and cultivation-dependent measurement variability of algal biomass composition, Anal. Biochem. 452 (2014) 86–95.
[16] E. Koru, Earth food spirulina (Arthrospira): production and quality standards, in: Y. El-Samragy (Ed.), Food Addit, InTech, Rijeka 2012, pp. 191–203.
[17] M.E. Gerschwin, A. Belay, Spirulina in Human Nutrition and Health, CRC Press LLC, Boca Raton, FL, 2007.
[18] I.S. Chronakis, A.N. Galatanu, T. Nylander, B. Lindman, The behaviour of protein preparations from blue-green algae (*Spirulina platensis* strain Pacifica) at the air/water interface, Colloids Surf. A Physicochem. Eng. Asp. 173 (2000) 181–192.
[19] M.A. Devi, G. Subbulakshmi, K.M. Devi, L.V. Venkataraman, Studies on the proteins of mass-cultivated, blue-green algae (*Spirulina platensis*), J. Agric. Food Chem. 29 (1981) 522–525.
[20] J.C. Moore, J.W. DeVries, M. Lipp, J.C. Griffiths, D.R. Abernethy, Total protein methods and their potential utility to reduce the risk of food protein adulteration, Compr. Rev. Food Sci. Food Saf. 9 (2010) 330–357.
[21] M.D.C. Garcia, C.V.G. Lopez, F.G.A. Fernandez, C.S. Bustos, Y. Chisti, J.M.F. Sevilla, Protein measurements of microalgal and cyanobacterial biomass, Bioresour. Technol. 101 (2010) 7587–7591.
[22] J.C. Servaites, J.L. Faeth, S.S. Sidhu, A dye binding method for measurement of total protein in microalgae, Anal. Biochem. 421 (2012) 75–80.
[23] S.P. Slocombe, M. Ross, N. Thomas, S. McNeill, M.S. Stanley, A rapid and general method for measurement of protein in micro-algal biomass, Bioresour. Technol. 129 (2013) 51–57.
[24] M.G. Redinbaugh, R.B. Turley, Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions, Anal. Biochem. 153 (1986) 267–271.
[25] AOAC, Amino acids in feeds, Off. Method 994.12, 1997.
[26] J. Mosse, Nitrogen-to-protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content, J. Agric. Food Chem. 38 (1990) 18–24.
[27] D.B. Jones, Factors for converting percentages of nitrogen in foods and feeds into percentages of protein, USDA Circ. 183 (1931) 1–21.
[28] R. Tkachuk, Note on the nitrogen-to-protein conversion factor for wheat flour, Cereal Chem. 43 (1966) 223–225.
[29] R. Tkachuk, Nitrogen-to-protein conversion factors for cereals and oil-seed meals, Cereal Chem. 46 (1969) 419–423.

[30] J. Mossé, J.C. Huet, J. Baudet, The amino acid composition of wheat grain as a function of nitrogen content, J. Cereal Sci. 3 (1985) 115–130.
[31] J. Baudet, J.C. Huet, J. Mosse, Variability and relationships among amino acids and nitrogen in maize grains, J. Agric. Food Chem. 34 (1986) 365–370.
[32] M. Yamaguchi, Determination of the nitrogen-to-protein conversion factor in cereals, Mod. Methods Plant Anal. 1992, pp. 95–107.
[33] F. Mariotti, D. Tomé, P. Patureau Mirand, Converting nitrogen into protein — beyond 6.25 and Jones' factors, Crit. Rev. Food Sci. Nutr. 48 (2008) 177–184.
[34] S. Boisen, S. Bechandersen, B.O. Eggum, A critical-view on the conversion factor 6.25 from total nitrogen to protein, Acta Agric. Scand. 37 (1987) 299–304.
[35] S. Khanizadeh, D. Buszard, C.G. Zarkadas, Misuse of the Kjeldahl method for estimating protein content in plant tissue, Hortscience 30 (1995) 1341–1342.
[36] N. Sriperm, G.M. Pesti, P.B. Tillman, Evaluation of fixed nitrogen-to-protein (N:P) conversion factor (6.25) versus ingredient specific N:P conversion factors in feedstuffs, J. Sci. Food Agric. 91 (2011) 1182–1186.
[37] G.S. Diniz, E. Barbarino, J. Oiano-Neto, S. Pacheco, S.O. Lourenco, Gross chemical profile and calculation of nitrogen-to-protein conversion factors for nine species of fishes from coastal waters of Brazil, Lat. Am. J. Aquat. Res. 41 (2013) 254–264.
[38] L.M.L. Laurens, T.A. Dempster, H.D.T. Jones, E.J. Wolfrum, S. Van Wychen, J.S.P. McAllister, et al., Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries, Anal. Chem. 84 (2012) 1879–1887.
[39] S.O. Lourenco, E. Barbarino, J.C. De-Paula, L.O. da S.O. da S. Pereira, U.M. Lanfer Marquez, S.O. Lourenço, et al., Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds, Phycol. Res. 50 (2002) 233–241.
[40] C. Safi, M. Charton, O. Pignolet, F. Silvestre, C. Vaca-Garcia, P.-Y. Pontalier, Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors, J. Appl. Phycol. 25 (2012) 523–529.
[41] A. Derrien, L. Coiffard, C. Coiffard, Y. De Roeck-Holtzhauer, Free amino acid analysis of five microalgae, J. Appl. Phycol. 10 (1998) 131–134.
[42] C.V. Morr, Nitrogen conversion factors for several soybean protein products, J. Food Sci. 46 (1981) 1362–1367.
[43] C.V. Morr, Recalculated nitrogen conversion factors for several soybean protein products, J. Food Sci. 47 (1982) 1751–1752.
[44] Y.K. Chau, L. Chuecas, J.P. Riley, The component combined amino acids of some marine phytoplankton species, J. Mar. Biol. Assoc. UK 47 (1967) 543–554.
[45] N.L. Hockin, T. Mock, F. Mulholland, S. Kopriva, G. Malin, The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants, Plant Physiol. 158 (2012) 299–312.
[46] Q. Dortch, S.I. Ahmed, T.T. Packard, Nitrate reductase and glutamate dehydrogenase activities in *Skeletonema costatum* as measures of nitrogen assimilation rates, J. Plankton Res. 1 (1979) 169–186.
[47] F.W. Sosulski, G.I. Imafidon, Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods, J. Agric. Food Chem. 38 (1990) 1351–1356.
[48] J.A. Gerde, T. Wang, L. Yao, S. Jung, L.a. Johnson, B. Lamsal, Optimizing protein isolation from defatted and non-defatted Nannochloropsis microalgae biomass, Algal Res. 2 (2013) 145–153.
[49] A. Schwenzfeier, P.A. Wierenga, H. Gruppen, Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp. Bioresour. Technol. 102 (2011) 9121–9127.
[50] D. Shuuluka, J.J. Bolton, R.J. Anderson, Protein content, amino acid composition and nitrogen-to-protein conversion factors of *Ulva rigida* and *Ulva capensis* from natural populations and *Ulva lactuca* from an aquaculture system, in South Africa, J. Appl. Phycol. 25 (2012) 677–685.
[51] R.N. McArdle, J. Lasekan, R. Engel, P. Behrens, Protein and amino acid content of selected microalgae species, LWT Food Sci. Technol. 27 (1994) 249–252.
[52] G.S. Diniz, E. Barbarino, J. Oiano-Neto, S. Pacheco, S.O. Lourenço, Gross chemical profile and calculation of nitrogen-to-protein conversion factors for five tropical seaweeds, Am. J. Plant Sci. 2 (2011) 287–296.