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# The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five

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**Abstract** A global drive to source additional and sustainable biomass for the production of protein has resulted in a renewed interest in the protein content of seaweeds. However, to determine accurately the potential of seaweeds as a source of protein requires reliable quantitative methods. This article systematically analysed the literature to assess the approaches and methods of protein determination and to provide an evidence-based conversion factor for nitrogen to protein that is specific to seaweeds. Almost 95 % of studies on seaweeds determined protein either by direct extraction procedures (42 % of all studies) or by applying an indirect nitrogen-to-protein conversion factor of 6.25 (52 % of all studies), with the latter as the most widely used method in the last 6 years. Meta-analysis of the true protein content, defined as the sum of the proteomic amino acids, demonstrated that direct extraction procedures underestimated protein content by 33 %, while the most commonly used indirect nitrogen-to-protein conversion factor of 6.25 over-estimated protein content by 43 %. We therefore determined whether a single nitrogen-to-protein conversion factor could be used for seaweeds and evaluated how robust this would be by analysing the variation in this factor for 103 species across 44 studies that span three phyla, multiple geographic regions and a range of nitrogen contents. An overall median nitrogen-to-protein conversion factor of 4.97 was established and an overall mean nitrogen-to-protein conversion factor of 4.76. We propose that the overall median value of 5 be used as the most accurate universal seaweed nitrogen-to-protein (SNP) conversion factor.

**Keywords** Amino acid · Macroalgae · Meta-analysis · Nitrogen-to-protein factor · Protein · Protein determination · Seaweed

## Introduction

The fundamental role of nitrogen and protein in the nutrition, physiology and ecology of seaweeds has been a key research topic for decades (Dawes et al. 1974; Rosell and Srivastava 1985; Hurd et al. 1996; McGlathery et al. 1996; Harrison and Hurd 2001; Nelson et al. 2008; Angell et al. 2014). However, the nitrogen and protein content of seaweeds has more recently become a focus of applied research (Harnedy and FitzGerald 2011; Boland et al. 2012), in particular for applications where the biochemical composition of species must be well characterised. These applications range from human and animal nutrition and health (Fleurence 1999b), and fertilisers and plant growth stimulants (Craigie 2011; Sharma et al. 2014) to bioenergy (Neveux et al. 2014). Together, these studies have generated a significant database on the protein biochemistry of seaweeds across diverse disciplines. However, there are inconsistencies and potential inaccuracies in the methods used to determine protein content arising from the use of direct extraction procedures for the measurement of soluble protein and the indirect (proxy) method of protein determination using a nitrogen-to-protein (N-protein) conversion factor of 6.25 (N×6.25).

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Protein determination using direct extraction procedures employs multiple options for the extraction component and for the subsequent quantification of soluble protein (mainly the alkaline copper assay (Lowry et al. 1951) and the Coomassie Brilliant Blue assay (Bradford 1976)). Both the extraction of the protein and the quantification of the extracted soluble protein are susceptible to inaccuracies. First, protein extraction yields are generally low for seaweeds due to the presence of cell wall mucilages and phenolic compounds (Fleurence et al. 1995; Wong and Cheung 2001). Second, the initial method of protein extraction is not a standardised process and consequently varies between studies. For example, protein extraction procedures vary with the pre-treatment of the sample (raw, milled, freeze/thawed, enzymatic digestion etc.), the volume of water and exposure time used for the extraction of water-soluble proteins, the type and exposure time of buffer used, whether or not the protein is precipitated, the method of precipitation (e.g., trichloroacetic acid to supernatant ratio), centrifuge time and force and the type of standard used (Berges et al. 1993; Fleurence et al. 1995; Fleurence 1999a; Wong and Cheung 2001; Barbarino and Lourenco 2005; Wong et al. 2006). These direct extraction procedures differ in efficiency in their own right and there is also an influence of the chemical and morphological features of the seaweeds themselves (Barbarino and Lourenco 2005). For example, tough leathery brown seaweeds may be more resistant to certain extraction procedures compared to seaweeds with soft thalli. Finally, irrespective of the extraction procedure, the main methods for quantifying protein in the extract are colorimetric assays (Bradford and Lowry assays) and these methods are also subject to interference from a number of factors depending on the biochemistry of the seaweed (Lowry et al. 1951; Compton and Jones 1985; Crossman et al. 2000). For example, the Bradford assay can underestimate protein in plant tissues rich in phenols and phenolases (Mattoo et al. 1987), which includes many brown seaweeds. Taken together, the number of unique combinations of extraction procedure, colorimetric assays and type of seaweed substrate leads to considerable variation in the quantitative determination of protein.

In contrast to the technical issues related to the direct extraction of protein, the determination of the total nitrogen content does not require any extraction of material and is simple, inexpensive and easily reproducible. Total nitrogen in tissue is determined mainly using either the Kjeldahl method (or a variation thereof) or through combustion using CHN analysers. While the methods for quantifying total tissue nitrogen content are less variable than the direct extraction procedures, the fallibility of this approach is the conversion factor then used to calculate the total protein. The traditional conversion factor of 6.25, which is used as the standard factor for seaweeds and many other materials (Mariotti et al. 2008), assumes that the total protein constitutes 16 % (100/6.25) nitrogen and, more erroneously, also assumes that all nitrogen is in the form of protein. In reality, all plant material including algae has significant sources of non-protein nitrogenous material such as chlorophyll, nucleic acids, free amino acids and inorganic nitrogen (e.g., nitrate, nitrite and ammonia) (Lourenço et al. 1998; Naldi and Wheeler 1999; Lourenço et al. 2004). This can, therefore, lead to an over-estimate of protein contents in seaweeds when the 6.25 conversion factor is applied (Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013). As a result, many studies have determined specific N-protein factors for commercially important terrestrial plants (Mossé et al. 1985; Mossé 1990; Sosulski and Imafidon 1990; Yeoh and Wee 1994; Yeoh and Truong 1996), fungal material (Danell and Eaker 1992; Fujihara et al. 1995), microalgae (Lourenço et al. 2004) and even for seaweeds (Aitken et al. 1991; Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013). However, these published factors are seldom used for seaweeds with most authors reverting to the traditional conversion factor of 6.25. Failing to implement a specific factor has the potential to cause economic losses, as it has threatened to do with established industries such as dairy (Mariotti et al. 2008). Therefore, recalibrating with a universal seaweed-specific factor when the seaweed industry is relatively in its infancy, could avoid economic losses in the future.

N-protein conversion factors are based on the quantification of total amino acids which is considered to be the most accurate way of determining protein (Heidelbaugh et al. 1975). Conversion factors have been calculated using two different methods. The first, which is referred to as $k_A$, uses the known molecular proportion of nitrogen of each individual amino acid, determined by quantitative amino acid analysis, to quantify the overall proportion of nitrogen in the total amino acid pool (Mossé et al. 1985; Mossé 1990). Although this method takes into account the specific amino acid profile of the material, it will overestimate the conversion factor if it is applied to total nitrogen content as it does not take into account the non-protein nitrogen. For this reason, conversion factors have also been calculated using another method, referred to as $k_P$, which is based on the ratio of the total amino acids to total nitrogen determined using independent methods (Mossé et al. 1985; Mossé 1990). Although $k_P$ takes into account non-protein nitrogen, it relies on the assumption that the total amino acid analysis is a true determination of protein. However, amino acid analyses may underestimate protein contents due to the partial or full destruction of some amino acids during hydrolysis (in particular, cysteine, tryptophan, methionine and serine) as well as the use of a single hydrolysis time that cannot guarantee the complete hydrolysis of certain amino acids without the destruction of others (Darragh and Moughan 2005). As a result of these inaccuracies, it has been suggested that $k_P$ will underestimate the true conversion factor (Mossé 1990), although some authors argue, for algae at least, that free amino acids also analysed in the process compensate

for the amino acids lost during hydrolysis (Lourenço et al. 2002). Therefore, because seaweeds typically contain high concentrations of non-protein nitrogen, the most accurate way for estimating the nitrogen-to-protein conversion factor is the determination of protein by total amino acid analyses and the independent determination of total N (Lourenço et al. 2002; Diniz et al. 2011).

Despite the established science for the calculation of N-protein conversion factors and much evidence to suggest that high concentrations of non-protein nitrogen are common for seaweeds, only a few empirical studies have calculated seaweed-specific nitrogen to protein conversion factors—all of which were lower than 6.25 (Aitken et al. 1991; Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013). Aside from these studies, the use of the N-protein factor of 6.25 remains the default factor for seaweeds. Similarly, authors continue to report direct extraction procedures and quantification of soluble protein in the literature despite these being highly variable and generally perceived to underestimate the content of protein in seaweeds (Crossman et al. 2000; Barbarino and Lourenco 2005; Shuuluka et al. 2013).

There is, therefore, a strong rationale for synthesising the body of data in the literature on the protein content of seaweeds to provide the simplest and most accurate standardised method for determining the content (proportion of dry weight) of protein in seaweed biomass. The aim of this meta-analysis is to quantitatively list the methods used in the literature, assess their suitability in quantifying protein and recommend the most appropriate method to determine protein in seaweeds. To do this, we consolidate available nitrogen and total amino acid data to calculate seaweed-specific N-protein conversion factors and analyse associations between these and the critical variables of taxonomic groups, geographic regions, cultivated and wild harvested seaweeds, and internal N content. The overarching goal of this meta-analysis is to determine if there is an acceptable universal seaweed-specific conversion factor, and if so, provide a justifiable value.

## Materials and methods

### Literature search

To retrieve a large number of original research articles that reported the protein or amino acid content in seaweeds, we searched the Web of Science core collection (1945–present) on 4 November 2014 using the following search string for terms in the title, key words or abstract: ((protein* OR amino*) AND (nutrition* OR nitrogen* OR lipid* OR carbohydrate* OR nutrient* OR biochemical* OR aquaculture*) AND (macroalga* OR seaweed*) NOT (enzyme* OR mycosporine*)). This search string was determined to be the most efficient at reducing the number of irrelevant articles while maintaining a large number of relevant articles. No constraints on the year of publication or the language of publication were imposed on the data base search. In addition, 17 articles that were not found by this search string were also included in the meta-analysis (see Supplementary Appendix for all articles included in meta-analysis).

To ensure that we only included articles that met our aim, we screened the results by reading the title, abstract and materials and methods to exclude those articles that did not contain a measure of protein or amino acids of unprocessed seaweed. We did not have access to 23 of the 259 articles that remained after the first screening. These were not examined further. We recorded the number of articles included and excluded according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement (see Fig. 1) (Moher et al. 2009).

### Extraction of qualitative data

For each article included in the meta-analysis (236 articles), the following qualitative information was recorded: (1) the year of the study; (2) the journal of publication; (3) the discipline of the article; (4) the phylum, genus and species of each seaweed analysed; (5) the geographic region of the seaweed (tropical, temperate or polar); (6) whether the seaweed was wild harvested or from a cultivation system; (7) the method used to determine the protein content in each sample; (8) the method used to determine the total tissue nitrogen content in each sample (if measured); (9) whether the total amino acids were measured and (10) the units in which the amino acids were reported.

Articles were divided into eight scientific disciplines, as defined in Table 1. Tropical regions were defined by the Tropic of Cancer (23° 26 16 N) and Tropic of Capricorn (23° 26 16 S), Polar Regions by the Arctic Circle (66° 33 44 N) and the Antarctic Circle (66° 33 44 S), and temperate regions between these latitudes. Wild harvested seaweeds were defined as any seaweed that was harvested from natural seawater (including sea-ranched seaweed) and cultivated seaweeds as any seaweed that were cultivated in an artificial land-based system. The protein determination methods that involved the extraction of protein were grouped together as 'extraction' methods and the method of protein quantification for each was recorded. The total amino acid (TAA) content as a protein determination method was defined by (1) the article reported the sum of amino acids as a proportion of dry weight and (2) there was no other method of protein determination. Only those articles which met these criteria were considered to have used TAA as a protein measurement. However, all amino acid data was used in the quantitative section of this meta-analysis, irrespective of whether it was used as the primary method for determining protein in an article (36 articles also

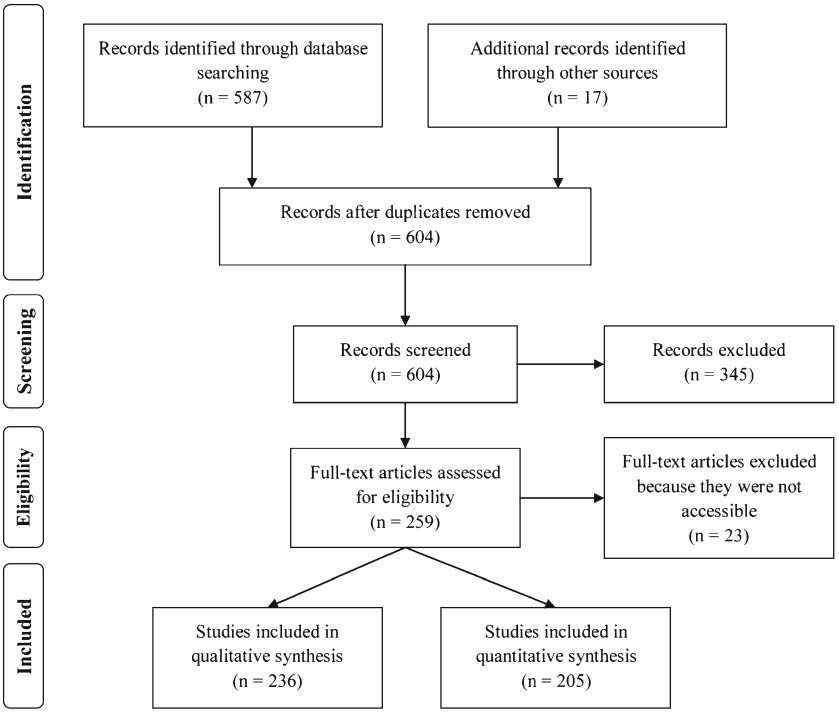


**Fig. 1** Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) literature search flow diagram (Moher et al. 2009)

reported TAA data in addition to the 14 which used TAA content as a protein determination method).

### Extraction of quantitative data

The following quantitative information was recorded: (1) the protein content in % dry weight (DW), (2) the tissue nitrogen content in % DW and (3) the total amino acid content in % DW (see Table 2 for definition of terms). When this data was only presented in a figure in an article, it was obtained using the software DataThief III (Tummers 2006). All measurements were converted to % DW. Measurements expressed in terms of fresh weight or ash-free dry weight were converted to % DW using moisture and ash contents, respectively. Protein measurements expressed as moles of nitrogen per unit biomass were converted using Eq. 1, assuming a protein nitrogen content of 16 % (Naldi and Wheeler 1999). Amino acid measurements expressed as moles per unit biomass were converted to % DW by using the sum of the molecular weight of individual amino acids. Total amino acid measurements expressed as % protein were converted into % DW using Eq. 2.

$$\text{Protein}\ (\%\ \text{DW}) = \frac{\text{Protein}\ (\text{mol N g}^{-1}\ \text{DW}) \times 14.007}{0.16} \quad (1)$$

$$\text{Total amino acids (TAA)}\ (\%\ \text{DW}) = \frac{\text{TAA}\ (\%\ \text{protein}) \times \text{Protein}\ (\%\ \text{DW})}{100} \quad (2)$$

If a measurement could not be converted to % DW, it was not included in the quantitative analysis of this meta-analysis.

**Table 1** Defining criteria for allocating articles to a particular scientific discipline in qualitative assessment of literature

| Discipline | Definition |
|---|---|
| 1. Analytical | The study's main aim was to test analytical methods |
| 2. Cultivation/bioremediation | The study investigated the culture of seaweed using either seawater or waste water |
| 3. Biochemical profiling | The study's main aim was to report biochemical profiles of seaweed in a non-physiological context |
| 4. Ecological | The study had an ecological context with its main aim to examine interactions between seaweed and other organisms |
| 5. Experimental/physiological | Any study which cultivated seaweed under experimental conditions where certain parameters were manipulated or where wild harvested seaweed was physiologically examined |
| 6. Feeding trial | Any study where the seaweed was used as a feed or feed ingredient in a feeding trial |
| 7. Protein extraction | The study's main aim was to test different protein extraction methods |
| 8. Protein digestibility | The study's main aim was to test the digestibility of protein from seaweeds |

**Table 2** Definition of commonly used terms in this article

| Term | Definition |
|---|---|
| Protein content | The protein as a percentage of dry weight measured by any method |
| Total nitrogen content | The nitrogen content as a percentage of dry weight measured by any method |
| Extraction procedure | Protein content measured by extracting soluble protein and quantifying it with a colometric assay (mainly Bradford and Lowry) |
| N×6.25 | Protein content determined by multiplying the nitrogen content by a factor of 6.25 |
| TAA | Protein content measured by quantifying and summing the proteomic amino acids (up to 20 amino acids, but most often 18 amino acids with cysteine and tryptophan excluded) |
| N-protein factor | The ratio of protein as measured by TAA to total nitrogen content |
| TAA N | The concentration of nitrogen in the TAA fraction of the biomass (in g N 100 $g^{-1}$ TAA, see Eq. 3) |
| Non-TAA N | The concentration of nitrogen in the non-TAA fraction of the biomass (in g N 100 $g^{-1}$ TAA, see Eq. 4). |

For quantitative analysis between determination methods, all available data was used for N×6.25 and TAA methods—not just data from measurements where the respective method was used to officially determine protein. This meant that all the nitrogen content data was multiplied by 6.25 to obtain the quantitative N×6.25 data and all TAA (% DW) were used for the quantitative TAA data.

N-protein factors—the ratio of TAA content (% DW) to total nitrogen content (% DW)—were calculated from two different data sets in this meta-analysis for different reasons. First, as a way to compare direct extraction procedures with TAA analysis, N-protein factors were calculated for all measurements of protein determined using both direct extraction procedures and TAA analysis which had a corresponding tissue nitrogen content measurement. This included TAA measurements which were not originally used to determine protein content but were reported along with nitrogen and/or protein content. Second, N-protein factors were also calculated just for TAA data as this method is considered the most accurate method for determining protein (Heidelbaugh et al. 1975). These N-protein factors were calculated only from the 5th to 95th percentile as not to represent extreme values and were used to determine seaweed-specific N-protein factors for an applied use and for correlations with internal N content. However, to determine seaweed-specific N-protein factors for an applied use, these N-protein factors were calculated using the means of each species so as not to over represent those species which had large numbers of measurements. However, for correlations with internal N content, raw N-protein factors (from the 5th to 95th percentile) calculated from all individual TAA data were used instead of the means of each species.

The concentration of nitrogen in both the TAA and non-TAA fractions were calculated for all seaweeds from which N-protein factors were calculated (with the exception of the data from five studies which did not report individual amino acid contents, $n=29$ individual measurements and $n=2$ species excluded). Furthermore, as with N-protein factors, the concentration of nitrogen in TAA and non-TAA acid fractions are reported based on the means of each species (as explained in the previous paragraph). The concentration of TAA nitrogen and non-TAA nitrogen are expressed as g N $(100\ g)^{-1}$ TAA and were calculated using Eqs. 3 and 4, respectively, where $D_i$ is the concentration of nitrogen in the $i$th AA per 100 g DW, $AA_i$ is the concentration of the $i$th AA per 100 g DW and total N is the total concentration of nitrogen per 100 g DW.

$$\text{TAA N} = \frac{\sum D_i}{\sum \text{AA}_i} \times 100 \tag{3}$$

$$\text{non-TAA N} = \left(\frac{\text{Total N}}{\sum \text{AA}_i}\right) \times 100 - \text{TAA N} \tag{4}$$

## Reporting of results and statistical analysis

All extracted quantitative and qualitative data were recorded in one Microsoft Excel 2007 spreadsheet with each column representing the qualitative and quantitative questions listed above and each row representing a unique measurement. Pivot tables were used to extract the qualitative meta-data. Qualitative data are presented in pie charts, bar graphs and tables. The % DW measurements of the major methods of protein determination (extraction, N×6.25 and TAA) and N-protein factors are presented using box and whisker plots overall, between phylum, between region and between cultivated and wild harvested seaweed using Statistica 12 (StatSoft Inc.). To standardise for nitrogen content, the methods of protein determination were also compared overall using box and whisker plots of N-protein factors. Boxplots were also used to compare the values and variation in TAA N and non-TAA N between- and within-species.

For all quantitative data, medians, means, inter-quartile ranges, 5th/95th percentile ranges and standard deviations were calculated using Statistica 12 and Microsoft Excel 2007.

As the data set for any specific combination of treatments was unbalanced, multivariate PERMANOVAs (PRIMER 6 & PERMANOVA+, PRIMER-E Ltd., UK) were used to analyse the effect of determination method on protein content (% DW) between taxonomic groups (green, brown and red seaweeds);

regions (temperate, tropical and polar) and cultivated vs. wild harvested seaweed. Differences in N determination method; N-protein factors between extraction and TAA methods; differences in N-protein factors between taxonomic groups (green, brown and red seaweeds), regions (temperate, tropical and polar) and cultivated vs. wild harvested seaweed were also all analysed using multivariate PERMANOVAs. Finally, differences in non-TAA N between the taxonomic groups were also analysed using multivariate PERMANOVAs. N-protein factors of extraction and TAA methods were also each compared to 6.25 using one-sample *t* tests (Statistica 12; StatSoft Inc.).

Correlations were made between N content and N-protein factor using Statistica 12 (StatSoft Inc.) for all data as well as all combinations of taxonomic group, region and wild harvested or cultivated. A separate correlation was also made between N content and N-protein factor for data in Angell et al. (2014).

## Results

Our systematic approach retrieved 604 articles that were potentially relevant to the meta-analysis (Fig. 1). Of these, 345 articles did not have any measure of protein or amino acids of unprocessed seaweed in the title, abstract or materials and methods and were excluded from the meta-analysis. The remaining 259 articles were read in full to extract the relevant qualitative and quantitative data; however, 23 of these could not be retrieved as full text articles and were excluded. This resulted in a total of 236 articles. Of these, 31 articles had quantitative data that could not be standardised as % DW and were therefore only used in the qualitative section (Fig. 1). Reasons for this included measurements in wet weight with no moisture content reported ($n$=17); no direct reporting of quantitative data ($n$=7); measurements in ash-free dry weight with no ash content reported ($n$=4, however, one of these was still used in the calculation of N-protein factors); amino acids reported as g 16 $g^{-1}$ N with no N content reported ($n$=1); amino acids reported as μmol $g^{-1}$ DW with no individual amino acid contents reported ($n$=1) and amino acids reported as % TAA with no TAA content reported ($n$=2, however, one of these had usable protein content data). In addition to these, the method of protein determination could not be retrieved for one article. The raw data set generated from this review has been made open access (Angell et al. 2015).

### Qualitative results

The 236 articles which were included in the meta-analysis could be divided into eight disciplines (Fig. 2a) and were published across 90 journals. The major disciplines were biochemical profiling studies (44 %, $n$=102); feeding trials (20 %, $n$=46); experimental/physiological studies (19 %, $n$=44); ecological (7 %, $n$=16) and cultivation/bioremediation

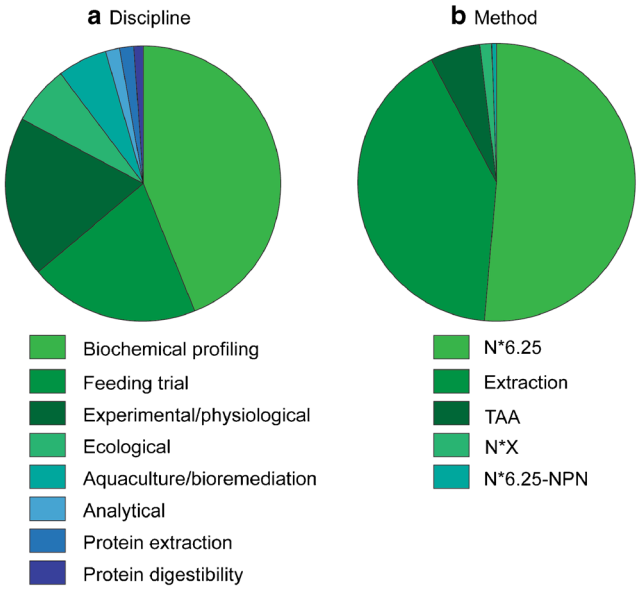


**Fig. 2** Proportion of **a** papers in different disciplines and **b** the methods used to determine protein in this review. Pie chart legends are listed in descending order of importance

(6 %, $n$=14). The majority of the articles were found in the *Journal of Applied Phycology* (12 %, $n$=29); *Food Chemistry* (7 %, $n$=16); *Aquaculture* (6 %, $n$=15); *Journal of Phycology* (6 %, $n$=13); *Botanica Marina* (5 %, $n$=12); *Marine Ecology Progress Series* (3 %, $n$=7); *Aquaculture Research* (3 %, $n$=6) and *Ecology* (2 %, $n$=5), representing 44 % of all articles. The remaining journals ($n$=82) had four or less articles with the majority having only one article ($n$=51).

Overall, five broad methods of protein determination were found: multiplying tissue nitrogen content by 6.25 (N×6.25), protein extraction and quantification of soluble protein (via the Bradford, Lowry, bicinchoninic acid assay (BCA) and UV absorption methods), quantification of total proteomic amino acids (TAA), multiplying tissue nitrogen content by a unique factor determined specifically for seaweeds (N×X) and N×6.25 without including non-protein nitrogen (N×6.25—NPN). Overall, the most commonly used method was N×6.25 (52 %) followed by direct extraction procedures (42 %) and TAA (6 %) (Fig. 2b). Almost 25 % of studies measured amino acids; however, over 70 % of these studies determined protein using either N×6.25 or direct extraction procedures. Within the major disciplines, N×6.25 was the most common method used in biochemical profiling studies (64 %), cultivation/bioremediation studies (64 %) and feeding trials (76 %). On the other hand, direct extraction procedures were most common in experimental/physiological studies (86 %) and ecological studies (75 %) (Table 3). Most extracted protein was quantified using the Bradford method (55 %), followed by the Lowry method (31 %) and the BCA method (11 %), with other methods making up less than 5 %.

Overall, protein data were recorded for 1841 measurements from 382 species. Red seaweeds were the most studied

**Table 3** Number of papers within each discipline and the protein determination methods used. Note: some papers determined protein using more than one method

| | Extraction | N×6.25 | N×6.25-NPN | N×X | TAA | Total papers |
|---|---|---|---|---|---|---|
| Analytical | 1 | 2 | 0 | 1 | 2 | 4 |
| Cultivation/bioremediation | 4 | 9 | 0 | 0 | 2 | 14 |
| Biochemical profiling | 32 | 65 | 1 | 1 | 5 | 104 |
| Ecological | 12 | 2 | 0 | 0 | 2 | 16 |
| Experimental/physiological | 38 | 4 | 0 | 0 | 2 | 45 |
| Feeding trial | 11 | 35 | 0 | 1 | 1 | 47 |
| Protein extraction | 0 | 4 | 0 | 0 | 0 | 4 |
| Protein digestibility | 0 | 2 | 0 | 0 | 0 | 2 |
| Total | 98 | 123 | 1 | 3 | 14 | 236 |

taxonomic group (highest number of measurements—43 %, $n$=792), followed by green seaweeds (32 %, $n$=576) and brown seaweeds (25 %, $n$=459). Red seaweeds were also the most diverse study group with 86 genera compared to brown seaweeds (50 genera) and green seaweeds (22 genera). Within the red seaweeds, the most studied genera were *Gracilaria* (29 %, $n$=232); *Palmaria* (12 %, $n$=98); *Gelidium* (9 %, $n$=68); *Eucheuma* (8 %, $n$=62) and *Porphyra* (6 %, $n$=51), with all remaining genera each representing less than 3 % ($n$=81 additional genera). Within the brown seaweeds, the most studied genera were *Sargassum* (19 %, $n$=85); *Dictyota* (12 %, $n$=54); *Macrocystis* (11 %, $n$=49); *Laminaria*/*Saccharina* (9 %, $n$=40); *Fucus* (6 %, $n$=27) and *Padina* (6 %, $n$=25), with all remaining genera representing less than 5 % ($n$=33 additional genera). Within green seaweeds, the genus *Ulva* represented the vast majority of measurements (67 %, $n$=385), followed by *Chaetomorpha* (12 %, $n$=70) and *Codium* (6 %, $n$=33), with all remaining genera representing less than 4 % ($n$=19 additional genera).

The number of articles published according to our criteria increased with time (Figure S1). Although only a small number of studies ($n$=11) were retrieved before 1995, owing to limited electronic database entries. The counts of articles retrieved since 1995 are an accurate representation of the size of the field. In 2010 and 2011, the total number of articles published per year increased dramatically and has remained high until the present. Before the year 2000, direct extraction procedures were generally the most common method for determining protein in seaweeds. However, more recently, the N×6.25 method has become the most widely utilised method, particularly in the last 6 years (2009–2014). Over the last 3 years (2012–2014), there has been a slight increase in the use of TAA as a method for determining protein, however, this method still represents a small proportion of studies (17 % in 2014).

## Quantitative results—methods of protein determination

Overall, direct extraction procedures yielded the lowest protein contents and use of the N-protein conversion factor of 6.25 (N×6.25) resulted in the highest, while TAA content resulted in an intermediate measure of protein (Fig. 3a, PERMANOVA: Pseudo-$F_{2, 2616}$=504.16, $p$<0.01). Direct extraction procedures ($n$=945) had the lowest mean protein measure (7.78 % DW) compared to both N×6.25 (PERMANOVA pair-wise test: $t$=31.53, $p$<0.01) and TAA ($t$=9.52, $p$<0.01) methods. In contrast, those protein contents determined using N×6.25 ($n$=1411) had the highest mean protein measure (16.60 % DW) compared to extraction (PERMANOVA pair-wise test: $t$=31.53, $p$<0.01) and TAA ($t$=11.17, $p$<0.01) methods. Finally, where protein was determined by TAA ($n$=299), it was an intermediate mean value compared to the other methods (11.60 % DW—see statistics above), with the protein contents of 90 % (5th/95th percentile) of seaweeds between 3 and 27 %. Notably, the spread of the data for each method (standard deviations) were relatively similar for extraction and TAA (6.35 and 6.93 % DW, respectively) but higher for N×6.25 (7.91 % DW) (see Table S1 for all descriptive statistics relating to methods of protein determination).

Protein contents were higher when using N×6.25, followed by TAA and direct extraction procedures, irrespective of the taxonomic group (Fig. 3b, PERMANOVA: Pseudo-$F_{2, 2610}$=489.16, $p$<0.001); the region where the seaweed was collected (Fig. 3c, PERMANOVA: Pseudo-$F_{2, 2610}$=207.72, $p$<0.001) or whether the seaweed was wild harvested or cultivated (Fig. 3d, PERMANOVA: Pseudo-$F_{2, 2613}$=375.14, $p$<0.001).

### *Taxonomic groupings*

Between taxonomic groups (Fig. 3b), brown seaweeds had the lowest mean protein content, followed by red seaweeds and green seaweeds (PERMANOVA pair-wise comparisons: $p$<0.01). Relative to the overall mean brown seaweed protein content (10.00 % DW), red seaweeds had 33 % more protein (13.31 % DW) and green seaweeds had 45 % more protein (14.48 % DW). This pattern remained similar when taxonomic groups were standardised for wild harvested seaweeds—brown seaweeds with the lowest (10.00 % DW)

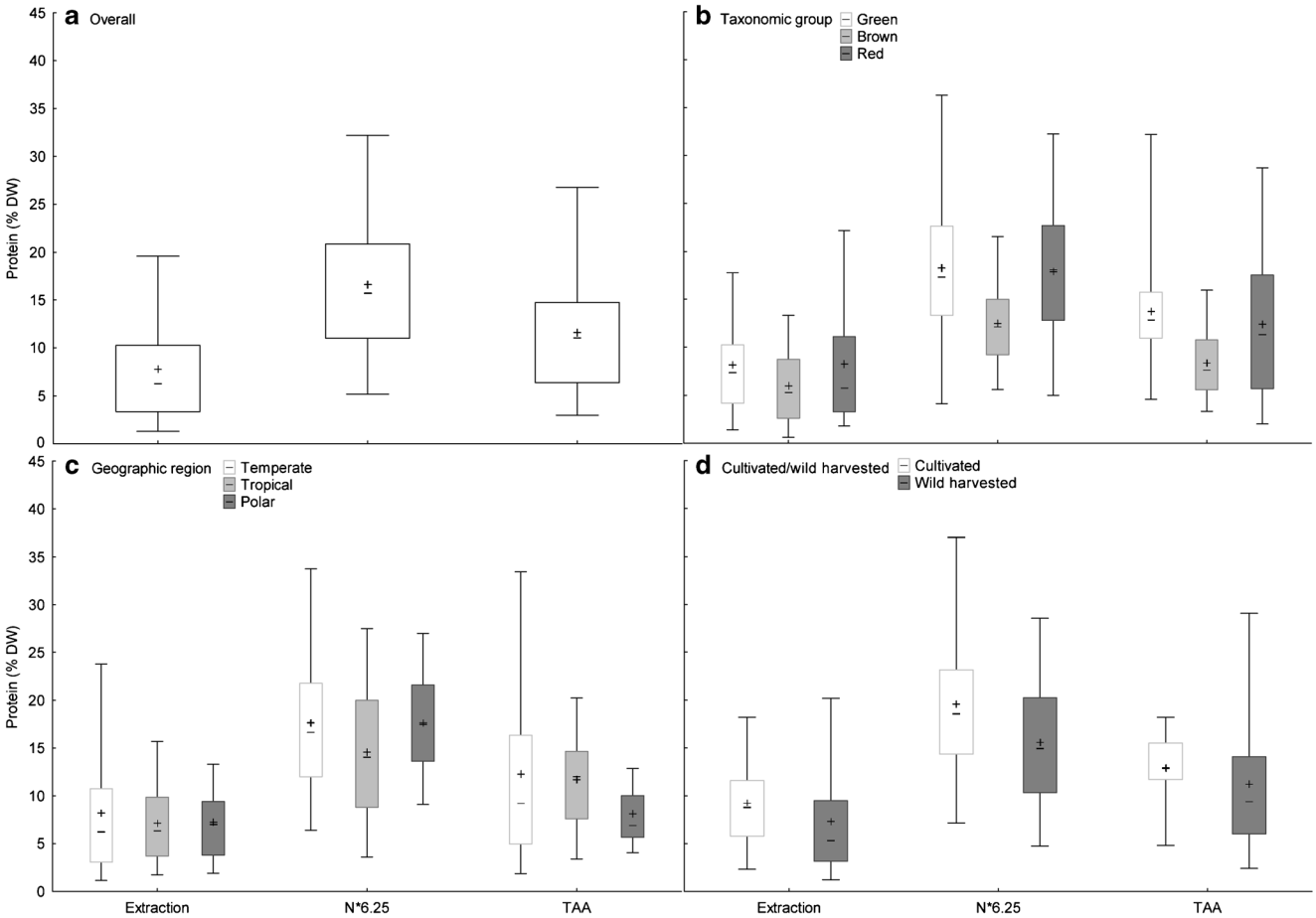


**Fig. 3** Quantitative protein measurements (% DW) of the papers examined in this review **a** overall, **b** among taxonomic groups, **c** among geographic regions and **d** among wild harvested and cultivated seaweed. *Dashes* represent medians, *crosses* represent means, *boxes* represent 25th percentiles and *whiskers* represent 5th/95th percentiles

(PERMANOVA pair-wise: $p<0.01$) compared to green seaweeds (12.73 % DW) and red seaweeds (13.11 % DW). Within each determination method, brown seaweeds maintained the lowest protein content, but red and green seaweeds only differed in mean protein content for TAA method (PERMANOVA pair-wise: $p<0.01$). The difference between brown seaweeds and green and red seaweeds was lowest for direct extraction procedures (means=8.13, 5.98, 8.24 % DW, respectively). However, for N×6.25 and TAA methods, red and green seaweeds had much higher mean protein contents relative to brown seaweeds (see Table S1 for all descriptive statistics). Based on the true proteomic (TAA) content, the green seaweeds had a 5th/95th percentile range of 4.6–32.2 % DW, the red seaweeds of 2.0–28.7 % DW and the brown seaweeds of 3.3–15.9 % DW.

*Geographic regions*

The relationships between geographic regions varied with the different protein determination methods. In a similar result to the taxonomic groupings, the three regions had the most similar mean protein contents when determined using direct extraction procedures (8.21, 7.11 and 7.21 % DW for temperate, tropical and polar, respectively), although extreme measurements were more variable for temperate seaweeds (c.f. whiskers in Fig. 3c). In contrast, when determined using the N× 6.25 method, mean protein content was slightly less for tropical seaweeds (14.57 % DW) compared to temperate (17.64 % DW) and polar (17.63 % DW—all of which were brown) seaweeds (PERMANOVA pair-wise comparisons: $p<0.001$). However, variation was similar between regions for the N×6.25 method (SD=7.98, 7.67 and 8.31 % DW for temperate, tropical and polar seaweeds, respectively). When determined using TAA methods, mean protein contents for tropical seaweeds (11.69 % DW) were lower than temperate seaweeds (12.29 % DW) and higher than polar seaweeds (8.14 % DW) (PERMANOVA pair-wise comparisons: $p<0.01$), with no significant difference between the mean measures of temperate and polar.

*Cultivated vs. wild harvested*

Protein contents were higher in cultivated seaweed (means= 9.26, 19.61 and 12.92 % DW for extraction, N×6.25 and TAA, respectively) compared to wild harvested seaweed (means=7.29, 15.58 and 11.22 % DW for extraction, N× 6.25 and TAA, respectively) for all three methods of determination (Fig. 3d, PERMANOVA pair-wise comparisons: $p<0.01$).

*N-protein conversion factor of different methods*

Both extraction (one-sample *t* test: $t_{537}=-19.85$, $p<0.01$) and TAA ($t_{279}=-16.15$, $p<0.01$) methods had mean N-protein conversion factors lower than 6.25 (Figure S2). However, N-protein factors calculated using direct extraction procedures were lower and more variable ($n=538$, mean=3.51, median=2.89, SD=3.20) compared to the TAA measurements ($n=279$, mean=4.69, median=4.87, SD=1.62) (PERMANOVA: Pseudo-$F_{1,816}=92.95$, $p<0.0001$).

**Determination of nitrogen**

Of the studies which measured total tissue nitrogen content in addition to protein and/or amino acids, 64 % used a variant of the Kjeldahl method and 34 % determined nitrogen through combustion. Determination by combustion had a higher mean (2.77 % DW) and smaller standard deviation (1.05 % DW) compared to determination by the Kjeldahl method (mean= 2.563 % DW, SD=1.47 % DW) (Figure S3, PERMANOVA: Pseudo-$F_{1,\ 1289}=32.76$, $p<0.001$).

**Nitrogen-to-protein conversion factors**

The nitrogen-to-protein conversion factors were determined for 110 species from 289 individual measurements (excluding within-article replication) but were calculated only for the 5th/95th percentile range (103 species, 260 individual measurements) (see Table S2 for all individual species N-protein factor data). Overall, the N-protein factors had a mean value of 4.76, a median of 4.97, an inter-quartile range of 3.83–5.68, a 5th/95th percentile range of 2.74–6.24 and a SD of 1.14 (Fig. 4a).

There was a significant albeit small difference in the mean N-protein factors between the different taxonomic groups (Fig. 4b, PERMANOVA: Pseudo-$F_{2,100}=3.34$, $p<0.05$). Green and brown seaweeds had lower mean and median N-protein factors (PERMANOVA pair-wise comparisons: $t=2.390$ and 2.259, $p<0.05$, means=4.49 and 4.56, medians= 4.68 and 4.81, for green [$n=26$] and brown [$n=35$] seaweeds, respectively) compared to red seaweeds ($n=42$, mean=5.10, median=5.31). There was no significant difference in mean N-protein factors between the three regions (temperate: $n=30$, mean=4.89, median=5.28, SD=1.06; tropical: $n=74$, mean=4.79, median=5.98, SD=1.14). There were only two samples from polar regions; however, these had noticeably lower N-protein factors (mean=3.04, median=3.04) (Fig. 4c). Finally, there was no significant difference in mean N-protein factor between cultivated and wild harvested seaweeds, although this was heavily weighted to wild harvested compared to cultivated seaweeds. Wild harvested seaweeds ($n=98$) generally had higher and more variable N-protein factors (mean=4.80, median=5.05, SD=1.15) than cultivated seaweeds ($n=6$, mean=4.25, median=4.42, SD=0.76) (Fig. 4d).

*Total amino acid versus non-total amino acid nitrogen*

Overall, the total amino acid content represented considerably more nitrogen (TAA N; mean=15.04 g N (100 g)$^{-1}$ TAA, median=15.04 g N (100 g)$^{-1}$ TAA) compared to the non-TAA nitrogenous components (non-TAA N; mean=7.51 g N (100 g)$^{-1}$ TAA, median=4.72 g N (100 g)$^{-1}$ TAA). However, there was considerably more variation in the concentration of non-TAA N (SD=7.32 g N (100 g)$^{-1}$ TAA) compared to TAA N (SD=0.71 g N (100 g)$^{-1}$ TAA) (Figure S4). For red seaweed, which was the only taxonomic group to have a significantly different N-protein factor, the non-TAA N was lower (mean=5.28 g N (100 g)$^{-1}$ TAA) compared to green and brown seaweeds (8.98 and 9.17 g N (100 g)$^{-1}$ TAA, respectively) (PERMANOVA pair-wise comparisons: $p<0.05$), although for brown seaweeds, this was not statistically significant.

*Correlations between N content and N-protein conversion factors*

Overall, there was no correlation between internal N content and N-protein factor (Table S3). However, there were significant correlations between various combinations of the categories. Many of these correlations were driven by Angell et al. (2014) which was the only study to measure N content and TAA content for a large number of individuals ($n=60$) at a species level over a large range of internal N contents. As the study focused on the tropical green seaweed *Ulva ohnoi*, this resulted in relatively strong negative relationships between internal N content and N-protein factor for all sub-groups which encompassed tropical, green or cultivated seaweed (see Table S3 for $r^2$ and $p$ values). However, there were still a number of other correlations, albeit weaker relationships, within other sub-groups. For example, brown seaweeds (all of which were wild harvested) showed a significant negative correlation as did red tropical seaweeds (all of which were wild harvested) and tropical wild harvested seaweeds. In contrast, temperate seaweeds and, more specifically, wild harvested temperate seaweeds had significant positive relationships between N content and N-protein factors, although these were

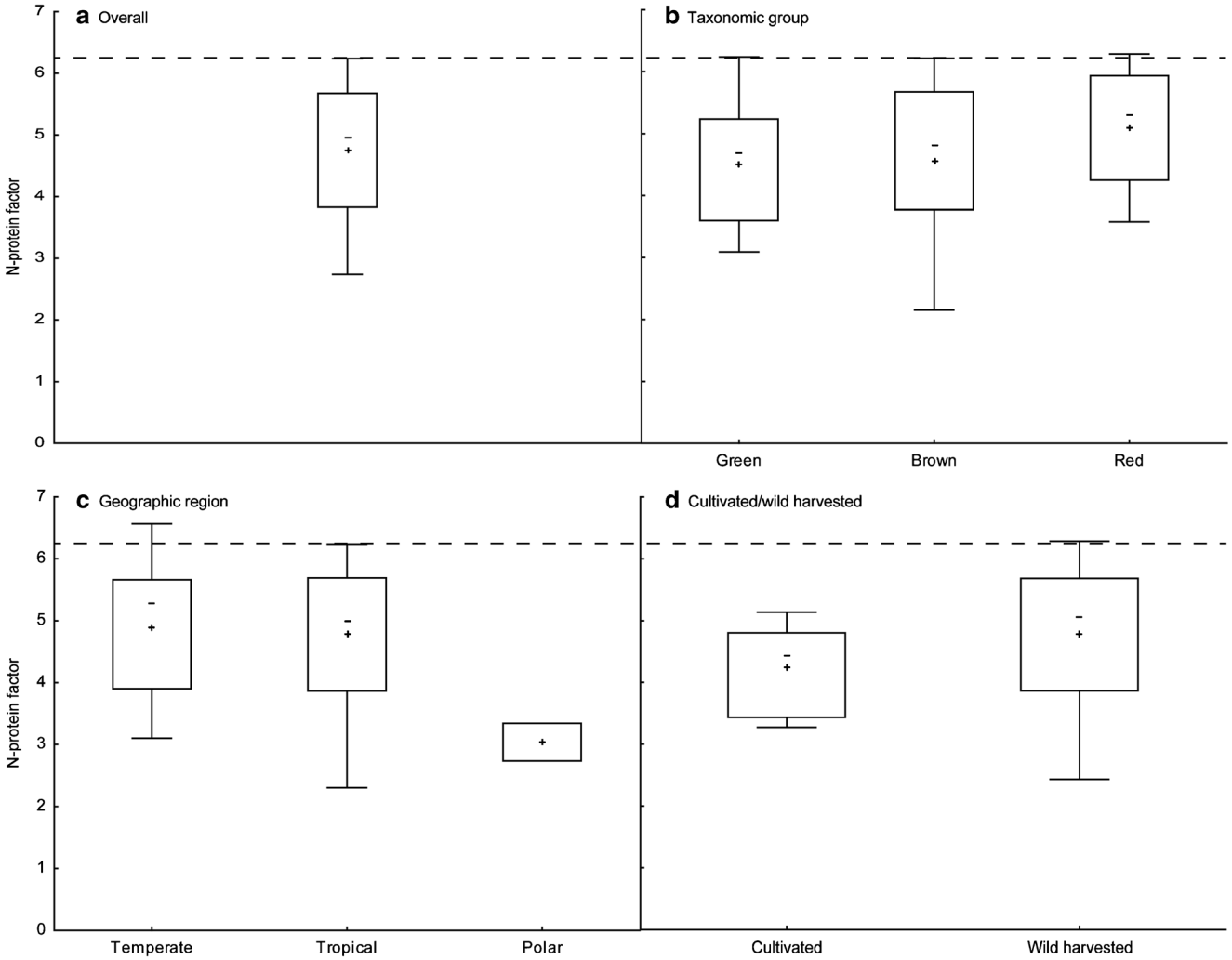


**Fig. 4** Nitrogen-to-protein conversion factors calculated from papers in this review **a** overall, **b** among taxonomic groups, **c** among geographic regions and **d** among wild harvested and cultivated seaweeds. *Dashes* represent medians, *crosses* represent means, *boxes* represent 25th percentiles and *whiskers* represent 5th/95th percentiles. *Dashed lines* indicate a conversion factor of 6.25

very weak ($r^2$=0.062 and 0.081, respectively) (see Table S3 for all correlations).

*Within-species variation in N-protein conversion factors*

There was considerable variance in N-protein factors within the green seaweed *U. ohnoi*. *U. ohnoi* had a mean value of 5.14, a median value of 5.17 and a SD of 0.47 (Figure S5a). Similar to the between-species results (Figure S4), total amino acids represented considerably more nitrogen (mean= 15.10 g N (100 g)$^{-1}$ TAA, median=14.86 g N (100 g)$^{-1}$ TAA) compared to non-TAA, nitrogenous components (mean=4.51 g N (100 g)$^{-1}$ TAA, median=4.48 g N (100 g)$^{-1}$ TAA). However, there was considerably more variation in the concentration of non-TAA nitrogen (SD= 1.57 g N (100 g)$^{-1}$ TAA) compared to TAA nitrogen (SD= 0.52 g N (100 g)$^{-1}$ TAA) (Figure S5b).

## Discussion

A resurgence of interest in the industrial applications of seaweeds has led to a large (~200–300 %) increase in the number of studies published per year examining protein data from 2009 to the present. However, there are considerable differences in approach between these studies used to determine protein. Only a limited number of studies in the field measured the true protein content using the sum of proteomic amino acids (<6 %) and the vast majority determined protein using either direct extraction procedures and the subsequent determination of soluble protein (42 %—mainly via Bradford and Lowry assays) or the indirect method of protein determination using the generic N-protein conversion factor of 6.25 translated from terrestrial animal and plant literature (52 %). The meta-analysis of the reported data for these methods demonstrates that direct extraction procedures generally underestimated protein content in seaweeds, and that the

6.25 N-protein factor over-estimated protein content in seaweeds relative to protein determined by the sum of proteomic amino acids (TAA). However, the true proteomic amino acid analysis remains an expensive and technical method that is seldom used to determine protein contents in seaweeds. Therefore, we suggest that a seaweed-specific N-protein factor calculated from total amino acid analyses offers a simple, relatively inexpensive and easily reproducible method for protein determination. A consolidation of all nitrogen and total amino acid data shows that 95 % of N-protein factors for seaweeds are lower than 6.25. On the base of this evidence, we propose a universal N-protein factor for seaweeds of 5 in place of the commonly cited factor of 6.25 to be used when TAA is not calculated.

### Methods of protein determination

The large majority of articles (96 %) analysed in this meta-analysis could be divided into the five main disciplines of biochemical profiling, feeding trial, experimental/physiological, ecological and cultivation/bioremediation studies. Of these, biochemical profiling, feeding trial and cultivation/bioremediation studies predominantly determined protein using the 6.25 N-protein factor, while experimental/physiological and ecological studies predominantly determined protein using direct extraction procedures. Further, over 70 % of articles ($n$=41) which measured amino acids still determined protein using either extraction or N×6.25 methods. The latter point speaks to a desire by authors to present protein data, where possible, in a standardised manner, and this concept should be taken into consideration when recommending a unified approach for studies across disciplines.

We found that the choice between the proxy N×6.25 method and an extraction procedure is linked to the discipline that the research falls under. While the disciplines that were primarily measuring protein for nutritional purposes predominantly used the N×6.25 method (66 % of the "nutritional" literature), the disciplines that examined the relative changes in protein content with respect to an experimental treatment or ecological process predominately used direct extraction procedures (82 % of the "physiological" and "ecological" literature). This suggests that the selection of the N×6.25 method may be biased when the purpose of reporting protein is to provide a nutritional assessment of the seaweed, although in the case of feeding trial studies, the choice of N×6.25 is surprising considering the importance of optimal protein levels in animal feeds. In contrast, studies that examined protein in a physiological or ecological context were more likely to have used an extraction procedure over the proxy N×6.25 method because of a focus on the relative, within-study differences in protein rather than total nitrogen. Indeed, it is often acknowledged that direct extraction procedures typically underestimate protein in seaweeds. However, even within a study, differences can arise between algal species due to species-specific extraction efficiency (Fleurence et al. 1995; Barbarino and Lourenco 2005) and quantification accuracy (Crossman et al. 2000).

There were some clear trends in relation to the methods that simplified the primary outcomes of this meta-analysis, that is, protein contents determined using direct extraction procedures underestimated protein and N×6.25 method over-estimated protein compared to the true value as the total content of the amino acids. These outcomes were true irrespective of whether seaweeds were categorised into taxonomic groups (greens, browns and reds); regions (temperate, tropical and polar) or whether the seaweed was wild harvested or cultivated. These results are in agreement with the limited number of empirical studies where this has been compared for seaweeds (Fleurence et al. 1995; Crossman et al. 2000; Lourenço et al. 2002; Barbarino and Lourenco 2005; Shuuluka et al. 2013). However, there were some differences between the protein determination methods within the various groupings of the categories. There was relatively little variability both overall and between taxonomic groups for direct extraction procedures, despite direct empirical evidence suggesting high variability in the efficacy of direct extraction procedures between different algal species (Fleurence 1999a; Crossman et al. 2000; Barbarino and Lourenco 2005). The determination by N×6.25 and TAA, which are both based on more standardised technical methods, showed more variation overall as well as more variation within green and red seaweeds, within temperate and tropical seaweeds and between cultivated and wild harvested seaweeds. However, these categories are broad and this variation may simply reflect the plasticity of nitrogen content in some seaweeds (Hanisak 1983; Naldi and Wheeler 1999; Harrison and Hurd 2001; Angell et al. 2014). This is supported by the higher variability in N-protein factors calculated for direct extraction procedures compared to those calculated for TAA.

Green and red seaweeds had a protein content 33–45 % higher than brown seaweeds, irrespective of the method of determination. Some of these differences were attributable to most of the brown seaweed analysed in this meta-analysis being from wild populations, however, even when standardised for this factor, brown seaweeds still had a lower protein content than green and red seaweeds—although this was reduced to 27–31 %. Indeed, cultivated seaweeds generally had higher protein contents compared to wild harvested seaweeds in this meta-analysis, irrespective of the method of protein determination. Unlike many natural environments, cultivated seaweeds are often not nutrient limited as they are grown in nutrient-rich water in land-based systems. In contrast, the described differences in protein content of seaweeds between geographic regions varied depending on the method of determination used, indicating the inability to compare

across methods for past work and the importance of using a universal approach to reporting protein. The lack of a defining pattern between seaweeds from different geographic regions across the different determination methods also suggests that this categorisation, unlike the others, does not have a strong link to protein physiology in seaweeds.

The highest protein content (as determined by TAA) was 32.2 % DW for green seaweeds, 28.7 % DW for red seaweeds and 15.9 % DW for brown seaweeds (95th percentile). Values beyond these are possible; however, they are rare and potentially questionable data unless they represent some restricted taxonomic groups under specific physiological conditions (Angell et al. 2014).

## Beyond 6.25—a seaweed-specific N-protein conversion factor

The traditional conversion factor of 6.25 overestimates protein contents in seaweeds. However, the use of a conversion factor is a standard approach that will remain a preferred method for the majority of studies because it is a simple means to estimate protein based on the measurement of nitrogen in the tissue. Empirical studies that have addressed the notion of seaweed-specific factors are restricted to a limited number of species ($n$=29) across a narrow geographic and nitrogen content range (Aitken et al. 1991; Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013).

Our synthesis of the available information in the literature calculated N-protein factors for a total of 103 species that spanned three taxonomic groups, multiple geographic regions and a range of physiological states. An overall median nitrogen-to-protein conversion factor of 4.97 was established and an overall mean nitrogen-to-protein conversion factor of 4.76. The mean N-protein factors for each of the categories were not statistically different for geographic regions (temperate, tropical and polar) and did not differentiate whether the seaweed was wild harvested or cultivated. This indicates that variation between species is far greater than any variation that exists within these categorisations and suggests that the use of specific N-protein factors for any of the geographic categories or whether the seaweed was wild harvested or cultivated is unnecessary. However, it is notable that between the taxonomic groups, red seaweeds had a higher mean N-protein factor (5.10) compared to green and brown seaweeds (4.49 and 4.59, respectively). Variation in N-protein factors stems from variation in the concentration of non-TAA nitrogen and variation in amino acid profiles (Mossé et al. 1985; Mossé 1990; Mariotti et al. 2008). While both of these factors varied among the seaweed species examined, the variation in non-TAA nitrogen varied considerably more than the variation in N content due to changing amino acid profiles (TAA N), supporting the former as the primary driver for between-species variation in N-protein factors. For red seaweeds, this non-TAA nitrogen was generally lower than in green and brown seaweeds and is likely the main reason behind their higher N-protein factors.

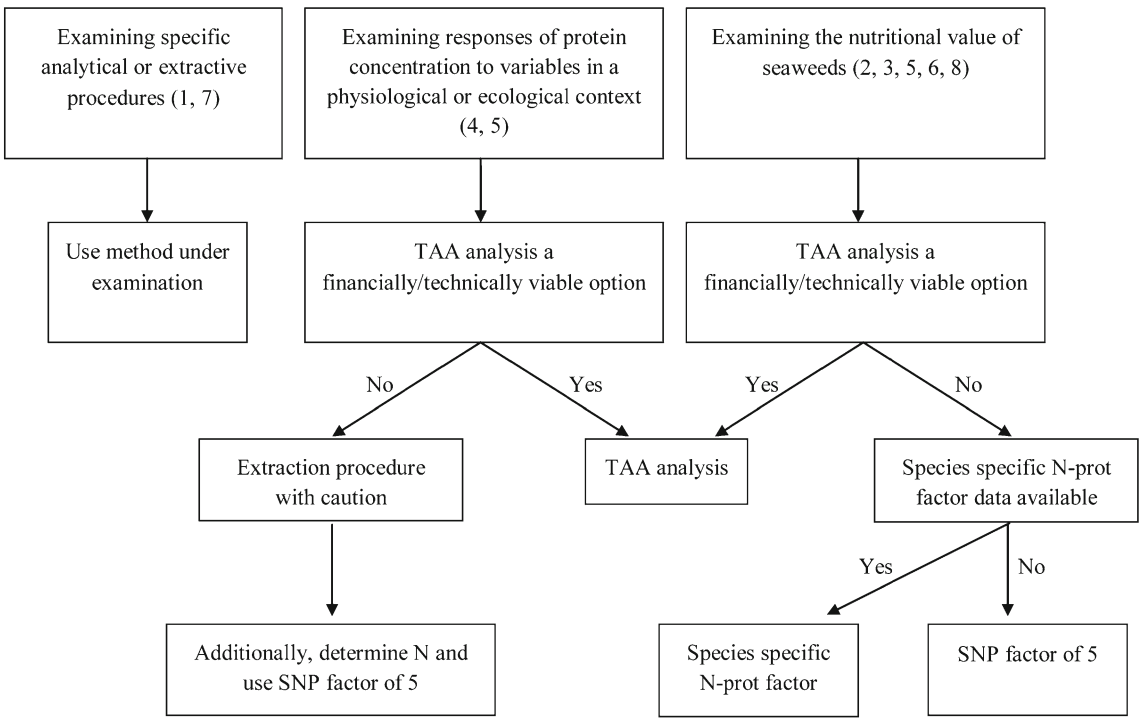


**Fig. 5** A decision tree for the selection of methods when determining seaweed protein content. *Numbers in parenthesis in the first row* refer to disciplines in Table 1

This result is in contrast to Lourenço et al. (2002) who calculated a mean N-protein factor for red seaweeds (4.92) that was lower than green (5.13) and brown (5.38). However, given the considerable variation between species and the smaller number of species examined by Lourenço et al. (2002) ($n$=19 compared to 103 here), these relative differences are likely a reflection of the local species used in that study. For simplicity, we consider that the variation in results and the small but significant difference between red and green and brown seaweeds is not of critical importance.

On a broad scale, there was little correlation between total N content and N-protein factor due to considerable between-species variation within the categories examined. However, if only a single species is examined, the correlation between total N content and N-protein factor can be pronounced. A study by Angell et al. (2014) provides insight into within-species variation in N-protein factors (for the green seaweed *U. ohnoi*), measuring total N and TAA content for a large number of individuals ($n$=60) over a large range of internal N contents. Within-species variation in N-protein factors was relatively high for *U. ohnoi* (SD=0.47, with 90 % of the data falling between 4.42–5.83), with variation primarily driven by non-TAA N, as it was at the higher taxonomic levels, and an N-protein factor that was negatively correlated with total N content. A decrease in N-protein factor with increasing N content is a result of the increased luxury consumption of N and the storage of this N in the form of both non-TAA N and amino acids rich in N such as arginine (Angell et al. 2014). Although there was some evidence for this in other taxonomic categories (namely brown wild harvested and red tropical subcategories: Table S3), additional study of within-species variation is required to confirm this pattern. However, it is likely that a negative correlation between total N content and N-protein factor will occur for any seaweed that has the capacity to store excess N during luxury consumption, of which many examples exist (Hanisak 1983; McGlathery et al. 1996; Naldi and Wheeler 1999; Taylor et al. 2006).

## Conclusion

In this meta-analysis, we calculated an overall median N-protein conversion factor of 4.97 (with a mean factor of 4.76) based on the ratio of total proteomic amino acids and total nitrogen. There were some minor differences between the red seaweeds (mean=5.10, median=5.31) and the green and brown seaweeds (means=4.49 and 4.56, medians=4.68 and 4.81, respectively). However, considering the large between- and within-species variation, we suggest that these categorical factors can be avoided to streamline the data and simplify the results. Therefore we propose that the overall median factor of 4.97 be rounded to 5 and used as the default seaweed N-protein (SPN) factor where accurate data on amino acids is not available. Although the median and mean are close, the median most accurately represents the variance in N-protein factors as it is less susceptible to outliers and skewed data. A SPN factor of 5 is also a straight forward conversion factor for calculations. This new factor can be applied retrospectively for previously presented N content data where 6.25 has been used but, more importantly, can be a standard for protein measurements in place of direct extraction procedures and N× 6.25, especially when reporting protein for nutritional purposes. Alternatively, order-, genus- or species-specific factors may also be applied (see Table S2), although we caution the use of factors outside the 5th/95th percentile range and those with low replication. In the case of many physiological or ecological studies where an interest lies in the changes to true protein rather than total N content (as a N-protein conversion represents), total amino acid analysis can be used to determine protein over direct extraction procedures for seaweeds, especially as there are many insights to be found in the changes to specific amino acids (Angell et al. 2014). We present a decision tree (Fig. 5) to demonstrate the benefits and limitations of alternative methods for protein determination in seaweed, with an overarching recommendation that the total N content is presented in addition to the protein content calculated using other methods.

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