LIMNOLOGY
and
OCEANOGRAPHY: METHODS

ASLO

Limnol. Oceanogr.: Methods 00, 2017, 00–00

doi: 10.1002/lom3.10186

Methodological biases in estimates of macroalgal macromolecular composition

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Abstract

Interest surrounding the use of macroalgae macromolecules for food products, biofuels, or other industrial applications is growing. As researchers search for macroalgae with especially high protein, lipid, carbohydrate or fibre content, the demand for a suite of standardized and unbiased methods for quantifying macroalgae macromolecules increases. Using data from available scientific literature, we evaluated the biases of the major methods used to determine macroalgal macromolecular content, as well as the sample drying methods employed. We found that drying at room temperature prior to analysis resulted in the highest estimates of protein and carbohydrate, and that freeze-drying provided the highest estimates of lipid. Using nitrogen content and the standard conversion factor to calculate protein in macroalgae (N × 6.25 method) overestimates protein content compared to protein assays such as the Bradford (1976) or Lowry (1951) assays. The Bligh and Dyer (1959) lipid extraction method was found to have a yield nearly two-fold higher than other standard methods. For carbohydrates, the By Difference and Prosky et al. (1984) methods provide estimates up to five-fold higher than other common methods used to determine carbohydrate and fibre. Based on these results we recommend using protein assays as opposed to nitrogen content assays to determine protein content, the Bligh and Dyer lipid extraction method for lipids, and the By Difference and Prosky method for carbohydrate and fibre, respectively.

Macroalgae range from the Arctic to Antarctic (Coyer 2007) and play an important role in Earth's ecosystems. Macroalgae are responsible for 5% of marine primary production and represent about two thirds of oceanic biomass (Smith 1981), making them an essential food source for herbivores and detritivores. Approximately 12,000 species of macroalgae have been identified across three different phyla: the Chlorophyta, Rhodophyta, and Ochrophyta, informally referred to as the green, red, and brown seaweeds (Rowan 1989; McHugh 2003; Guiry and Guiry 2016).

Humans harvest macroalgae for a variety of uses, including phycocolloid extraction (a carbohydrate used as a thickening agent), iodine extraction, aquaculture feed, and human consumption (Lee 1977; Lobban and Harrison 1994; Fleurence 1999; Nayar and Bott 2014; FAO 2016). There is interest in developing macroalgae for the production of biofuels, particularly Ochrophytes of the order Dictyotales (Gosch et al. 2012), and the Chlorophytes *Derbesia* sp. and *Oedogonium* sp. (Neveux et al. 2014, 2015). Many macroalgae are excellent sources of dietary fibre (Dawczynski et al. 2007) and the protein content of some species, like *Porphyra tenera* (nori) or *Palmaria palmata* (dulse) is comparable to that found in high-protein vegetables, such as soybeans (Mabeau and Fleurence 1993). Harvesting of macroalgae is a six billion US dollar per year industry that has increased by 50% in biomass production in the last 10 yr (Nayar and Bott 2014; FAO 2016).

Many surveys have been conducted on the major macromolecular composition (protein, lipid, carbohydrate, and dietary fibre) of macroalgae in order to identify strains suitable for human consumption, aquaculture, biofuels, and other applications. The quantification methods for each macromolecule and sample preparation methods each have their own biases, making it difficult to compare macromolecular composition across studies and effectively identify species with especially high protein, lipid or carbohydrate (dietary fibre) content. Total protein is commonly estimated from total nitrogen content by assuming that all nitrogen is associated with protein and that protein is 16% nitrogen by mass (multiplying total nitrogen by 6.25), or using a colorimetric assay that directly measures protein residues, such as the Bradford (1976) and Lowry (1951) protein assays. Using

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nitrogen content to estimate protein content with a conversion factor of 6.25 has been shown to provide an overestimate of protein (Lourenço et al. 2002; Angell et al. 2016). Total lipid content in macroalgae is most commonly determined by solvent extraction, however the best solvents and protocols to extract the majority of lipids from macroalgal tissues has not yet been determined. Different protocols and solvents recover more or less lipid depending on the relative polarity of solvents, sample types, and extent of tissue disruption (Randall et al. 1991; Manirakiza et al. 2001; Kumari et al. 2011). There is also a wide array of methods used to estimate carbohydrate and fibre in macroalgae that each measures a different fraction of the carbohydrate pool. Total carbohydrate methods include colorimetric assays such as the Anthrone method (Yemm and Willis 1954) and Dubois (1956) method, as well as the By Difference method, which calculates carbohydrate content by assaying ash free dry weight and subtracting the protein and lipid content of the samples (Merrill and Watt 1973). Fibre content is typically measured using the enzymatic gravimetric method of Prosky et al. (1984) or the Crude Fibre method (Henneberg and Stohmann 1859). In addition, there are several different methods used to dry specimens prior to macromolecular analyses. Inadequate drying of samples increases the moisture content and variability in estimates of macromolecular content as a percentage of dry weight. Furthermore, some drying methods may damage some macromolecules or affect extraction efficiency and detection (Chan et al. 1997; Wong and Cheung 2001).

Despite the knowledge that different methods may produce different results, there has been no systematic, quantitative study of how the use of different methods affects estimates of protein, lipid, and carbohydrate in macroalgae across studies. Note Angell et al. (2016) provide a methodological analysis of protein determination in macroalgae. Without knowledge of methodological biases associated with estimates of macromolecular composition it is difficult to pool or compare studies to identify macroalgal species with particularly high protein, lipid, or carbohydrate compositions that could be used for food products, biofuels, or other industrial applications. Here we compile a database of 125 studies of macroalgae macromolecules to determine if there are any systematic biases in estimates of protein, lipid, carbohydrate, and fibre as percent dry weight due to the methods employed. We use our results to recommend standard methods for determining macroalgal macromolecular composition.

Methods

Macroalgae macromolecular database

Macromolecular data was collected from the text, tables, and figures of 125 publications published between 1931 and 2016. These papers were gathered by searching Google Scholar from August 2015 to July 2016 using combinations of the following search terms: seaweed, macroalgae, Chlorophyta, Rhodophyta, Ochrophyta, green seaweeds, red seaweeds, brown seaweeds, macromolecular composition, biochemical composition, chemical composition, protein, fats, lipids, carbohydrates, fibre, nutritional value, nutritional composition, and calorie content. Image J (https://imagej.nih.gov/ij/) was used to extract data from figures, and AlgaeBase (http://www.algaebase.org/) was used to identify the current taxonomic status of all species documented. Macromolecular composition (protein, lipids, total carbohydrates, and fibre) as percent dry weight, the method used to estimate macromolecular content, and the method used to dry macroalgal samples was extracted from each paper. The database is available online at http://figshare.com (Fiset 2016), and includes a total of 1054 observations for protein, 796 for lipids, and 917 for carbohydrates from 364 species. The Chlorophyta have 348 macromolecular observations, the Ochrophyta 347 observations, and the Rhodophyta 492 observations. Macroalgal species that are widely distributed or of commercial interest, either as food sources or for their chemical properties, are over-represented in the database. The most commonly occurring species in the database are *Ulva lactuca* ($n = 58$), *Palmaria palmata* ($n = 45$), and *Gracilaria corticata* ($n = 21$). Only field samples were included in the database. Macroalgae grown in the lab are often exposed to conditions that alter macromolecular composition, making it more difficult to identify methodological biases. For instance, macroalgae exposed to high inorganic nitrogen concentration often have high protein content (Bird 1984; Shpigel et al. 1999; Viera et al. 2005; Angell et al. 2014).

Comparison of macromolecular methods: protein

Two methods for measuring protein content in macroalgae were compared. The most common method is to measure nitrogen content and multiply the nitrogen content by a standard conversion factor (6.25) to convert total nitrogen to protein. Nitrogen content is most commonly measured using either the Kjeldahl method (Kjeldahl 1883) or by thermal conductivity or infrared spectroscopy following combustion using a CHN analyzer. This method assumes that protein is 16% nitrogen by mass and that all measured nitrogen is protein. Values from sources that used conversion factors other than 6.25 were corrected to match the 6.25 conversion factor. The second most common set of methods uses an assay to detect peptide residues colorimetrically. The colorimetric protein methods include the Bradford assay (Bradford 1976) and modern enhancements of the classic Biuret test such as the Lowry assay (Lowry 1951) and Bicinchoninic Acid (BCA) assay (Lowry 1951; Smith et al. 1985). The Lowry assay and commercial variants of it, such as the Bio-Rad DC Protein Assay (Bio-Rad Laboratories), account for 76% of the peptide-based observations of protein as percent dry weight in our database. For our analyses, we refer to all these colorimetric peptide-detecting assays as Protein Assays.

Comparison of macromolecular methods: lipid

Lipid extraction methods were grouped into four categories Folch et al. (1957), Bligh and Dyer (1959), AOAC (1990), and Other. The Folch extraction method (Folch et al. 1957) involves extracting homogenized samples (assumed to be 80% water by mass or rehydrated to this condition) in 2 : 1 (v/v) chloroform–methanol at a 20 : 1 ratio of solvent:sample, partitioning this crude extract with water or a weak salt solution to achieve 8 : 4 : 3 (v/v/v) chloroform–methanol–water, and then removing aqueous contaminants. This biphasic system is then filtered to remove the homogenate and the organic phase is removed as a purified lipid extract. The Bligh and Dyer method is similar to the Folch method except that the initial organic extraction is performed with 1 : 2 (v/v) chloroform–methanol at a 3 : 1 ratio of solvent:sample, which is followed by the addition of chloroform to adjust the solvent to 1 : 1 (v/v) chloroform–methanol, and has an aqueous partitioning at a ratio of 2 : 2 : 1.8 (v/v/v) chloroform–methanol–water. Following either of these lipid extraction methods, the purified lipid extract is dried and total lipid content is generally determined gravimetrically. A small proportion of Folch lipid observations (3%) determined lipid content using the sulpho-phospho-vanillin method (Frings and Dunn 1970). There were also two AOAC lipid extraction methods (925.32 and 923.05) that were frequently used in this study, with the values produced by each pooled as "AOAC" (AOAC 1990). The AOAC 925.32 method involves an initial acid hydrolysis of samples at 100°C. This hydrolysate is then partitioned with ether and the organic ether phase is removed as a purified lipid extract. The AOAC 923.05 method consists of an initial extraction of homogenized samples with hot aqueous alcohol and ether, followed by filtering of the extract with sand and asbestos. Following each of the AOAC methods described above, the filtered, purified lipid extracts are generally dried and total lipid content is determined gravimetrically. The Other category includes methods that used petroleum ether (5% of total lipid observations), ethyl ether (6%), dichloromethane (0.6%), or other extraction solvents. The lipid data from four publications (Heiba et al. 1990; Jayasankar 1993; Kaliaperumal et al. 1994; Gokulakrishnan et al. 2015) were removed from the dataset prior to analyses (and were not included in the total observations count) due to consistently anomalously high values.

Comparison of macromolecular methods: total carbohydrate and fibre

We compare three methods that estimate total carbohydrate content in macroalgae: the Anthrone method (Yemm and Willis 1954), the Dubois method (Dubois et al. 1956), and the By Difference method (Merrill and Watt 1973). The Anthrone method uses sulfuric acid to hydrolyze complex carbohydrates into simple sugars, then glucose is dehydrated to hydroxymethyl furfural, which forms a green compound upon the addition of anthrone (Dreywood 1946; Yemm and Willis 1954). The Dubois method, also known as the phenol-sulfuric acid (PSA) method, is the most common total carbohydrate method (Brummer and Cui 2005). It hydrolyzes glucose with sulfuric acid, forming furan derivatives, which in turn produce a dark yellow complex upon the addition of phenol (Dubois et al. 1956; Brummer and Cui 2005). The By Difference method calculates the total carbohydrate content in samples by subtracting the percent dry weight of protein, lipid, and ash (which are measured using other techniques) from total dry weight and assuming the remaining mass is carbohydrate (Merrill and Watt 1973).

In addition to these three total carbohydrate methods, we also compared the Prosky and Crude Fibre methods (Henneberg and Stohmann 1859; Prosky et al. 1984). These two methods measure fibre, which are defined physiologically as the subset of carbohydrates that are not digested by the small intestine (Knudsen 2001). Fibre is mostly composed of plant cell wall polysaccharides, such as cellulose and lignin, and does not include simple sugars and starches, whereas total carbohydrates include fibre, sugars, and starches (Knudsen 2001; Chawla and Patil 2010). The Prosky method, equivalent to the AOAC 985.29 method (AOAC 1990), is similar to the By Difference method in that it assumes that carbohydrate is the remainder after other cell contents are quantified, but also includes an extraction of more labile carbohydrates (sugars and starches) and assumes the rest is fibre (Prosky et al. 1984). Enzymes are first added to digest polysaccharides into glucose, then ethanol is added to precipitate soluble dietary fibre, and then particulate material is separated, dried, and weighed. Total dietary fibre is calculated by subtracting the protein and ash values (calculated using other methods) from the weight of the dry residue. The Crude Fibre method (Henneberg and Stohmann 1859) involves boiling samples in weak sulphuric acid (simulating stomach digestion) followed by boiling in weak potassium hydroxide (simulating alkaline small intestine conditions) and the remaining particulate material is rinsed with acetone, dried, and weighed.

Comparison of macromolecular methods: drying methods

The effect of drying on estimates of protein, lipid, and carbohydrate content as percent dry weight was tested. Six categories of drying methods were examined: freeze-drying, oven-drying, room-temperature, sun-drying, and blotting. The majority of studies examined used oven-drying (57% of known observations), followed by freeze-drying (17%) and drying at room temperature (15%). Oven-dried samples were generally dried between 50°C and 100°C for 24 h or until they reached a constant weight. It was assumed that freeze-dried samples followed standard procedures and were sequentially pre-frozen, dried under vacuum while still frozen, and then stored at < 0°C until analysis (Labconco 2010).

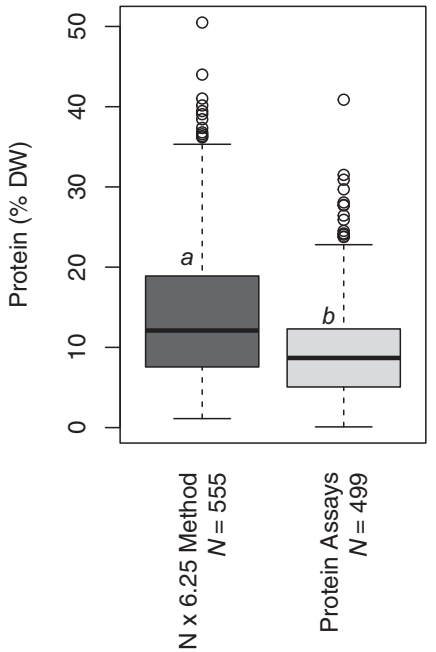

Fig. 1. Estimates of protein as percent dry weight (% DW) as a function of method shown as box plots to illustrate the distribution of observations. Median (± standard error) protein as percent dry weight is 12.10 ± 0.50 for the N × 6.25 method and 8.68 ± 0.36 for the Protein assays. Different letters above the boxes represent a statistically significant difference at $p < 0.05$ (Tukey HSD).

Samples that were sun-dried or dried at room temperature were often dried for several days or until they reached a constant weight. There does not appear to be a standardized method for blotting samples dry.

Statistical analyses

Median macromolecular composition as percent dry weight was calculated for protein, lipid, and carbohydrate and fibre for the full data set (all macroalgae) and for each of the phyla (Chlorophyta, Ochrophyta, and Rhodophyta). Standard errors on the medians were calculated by bootstrap resampling. One standard error is reported in the text and figures unless otherwise noted. ANOVAs were used to determine if macromolecular composition estimated using different methods were significantly different from each other at the $p \leq 0.05$ level using the full dataset, and nested ANOVAs were used at the phylum level. Tukey's honest significance difference (HSD) tests were used to determine which methods statistically differed from each other. All statistical tests were computed using R (Fox and Weisberg 2011; Wickham 2011; R Development Core Team 2016). Four highly calcified genera, *Halimeda*, *Amphiroa*, *Lithothamnion*, and *Calliarthron*, were not included in the analyses (or the species and total observations count) because their calcium carbonate content significantly reduces their macromolecular content on a percent dry weight basis (Steneck and Martone 2007). Observations of these species relative to less and non-calcified species using different methods could bias macromolecular composition as percent dry weight.

Table 1. Median protein as percent dry weight for macroalgae (full dataset) and the three phyla using the N content (N × 6.25) and Protein Assay methods. The two methods were significantly different for the full dataset and within each of the phyla (*t*-test, $p < 0.05$) except for the Ochrophyta (*t*-test, $p = 0.96$). *N* is sample size. SE is one standard error.

	N × 6.25		Protein assay	
Group	**Median ± SE**	***N***	**Median ± SE**	***N***
All	12.10 ± 0.50	555	8.68 ± 0.36	499
Chlorophyta	13.63 ± 0.85	121	10.02 ± 0.61	186
Ochrophyta	7.56 ± 0.29	177	8.20 ± 0.6	121
Rhodophyta	15.46 ± 0.81	257	6.37 ± 0.62	192

Results

Overview

Different methods used to estimate protein, lipid, carbohydrate, and fibre significantly impact apparent median macromolecular composition (ANOVAs, $p < 0.001$). The methods used to dry the samples also have an additional impact on estimates of protein, lipid, carbohydrate as a percentage of dry weight (ANOVAs, $p < 0.001$). Analyses were also conducted using ash-free dry weight data, but since there were no differences between the two, and that ash-free dry weight had a smaller sample size ($n = 667$ vs. $n = 1220$), data and results are only reported for the percent dry weight data.

Protein

Across the full dataset, protein as percent dry weight is significantly higher (12.10 ± 0.50 vs. 8.68 ± 0.36, *t*-test, $p < 0.001$) when estimated using nitrogen content (N × 6.25) compared to the more direct protein assays (Fig. 1). This result varies across the three macroalgae phyla (Table 1). For the Chlorophyta and Rhodophyta, protein as percent dry weight is significantly higher (13.63 ± 0.85 and 15.46 ± 0.81 vs. 10.02 ± 0.61 and 6.37 ± 0.62, respectively, *t*-tests, $p < 0.001$) when estimated using nitrogen content. However, for the Ochrophyta there is no significant difference in protein as percent dry weight across the two methods (7.56 ± 0.29 vs. 8.20 ± 0.62, *t*-test, $p = 0.96$).

The drying method used has a significant effect on estimates of protein as a percent of dry weight whether determined by nitrogen content or protein assay (ANOVAs, $p < 0.001$, Table 2). Protein as percent dry weight determined from nitrogen content is significantly higher for oven-dried samples than all other drying methods (Tukey HSD, $p < 0.001$) and is lowest for sun-dried samples. For the protein assays, protein as percent dry weight is highest for samples dried at room temperature and blotting, and lowest

Table 2. Median protein as percent dry weight as a function of both the protein determination and drying methods. Different letters represent statistically significant differences across methods at $p < 0.05$. *N* is sample size, SE is one standard error.

	N × 6.25		Protein assay	
Drying method	**Median ± SE**	***N***	**Median ± SE**	***N***
Freeze-dried	10.50 ± 1.13^{a}	96	8.00 ± 0.44^{c}	59
Oven-dried	15.31 ± 0.74^{b}	205	8.56 ± 0.46^{c}	326
Room temperature	11.40 ± 0.86^{a}	77	14.77 ± 1.50^{d}	40
Sun-dried	8.50 ± 0.50^{a}	36	0.61 ± 0.059^{e}	6
Blotted	10.45 ± 0.83^{a}	34	$11.04 \pm 0.65^{c,d}$	42

with sun drying. Although they generated the highest apparent yield, protein assay values from samples dried at room temperature also had the largest standard error and a relatively small sample size.

Lipid

The lipid extraction method with the highest lipid values as percent dry weight is the Bligh and Dyer method, followed by the Folch, AOAC, and Other methods (Fig. 2). The Bligh and Dyer method provides significantly higher lipid values as percent dry weight than all other methods (Tukey HSD, $p < 0.05$). The AOAC and Other methods, which had the lowest lipid values as percent dry weight, do not differ from each other (Tukey HSD, $p = 0.91$). The differences in lipid as percent dry weight across methods observed for the full dataset are also observed within the three phyla (nested ANOVAs and Tukey HSD tests).

Since the Bligh and Dyer method had the highest lipid yield for macroalgae, it was tested for the influence of the various drying methods. Freeze-drying and oven-drying generate the highest values of lipid as percent dry weight, and do not differ from each other (Tukey HSD, $p = 0.735$), but there was no significant effect of drying method on lipid estimates as percent dry weight using the Bligh and Dyer method (ANOVA, $p = 0.53$). We then tested the Folch method, since it had a larger sample size than the Bligh and Dyer method ($N = 312$ vs. $N = 188$). There is a significant effect of drying method on lipid estimates as percent dry weight using the Folch lipid extraction method (ANOVA, $p < 0.001$), where pairwise comparison tests revealed that freeze-drying and oven-drying differed and were significantly higher than blotting (Tukey HSD tests, $p < 0.001$). Similar to the Bligh and Dyer method, freeze-drying and oven drying had the highest lipid values and did not differ from each other (Tukey HSD, $p = 1.00$) using the Folch extraction method.

Carbohydrate and fibre

Fibre is a subset of the total carbohydrate pool. However, some of the fibre methods (for example: the Prosky method)

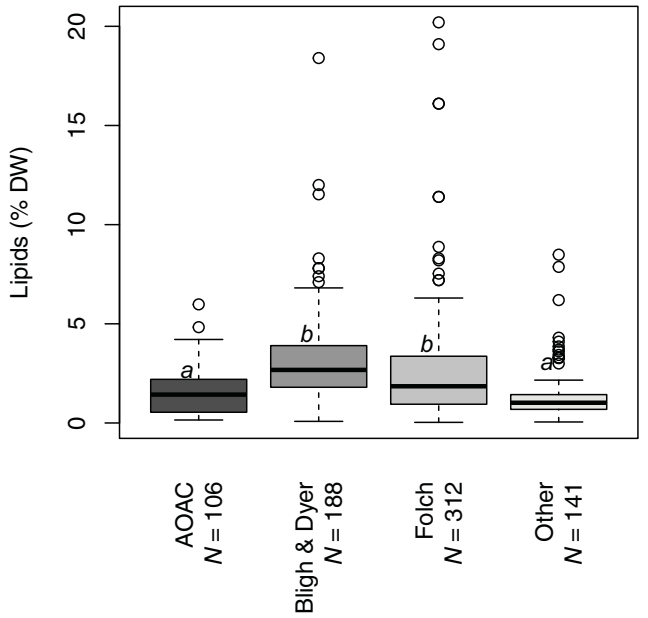

Fig. 2. Estimates of lipid as percent dry weight (% DW) as a function of lipid extraction method shown as box plots to illustrate the distribution of observations. Medians and standard errors for the AOAC, Bligh and Dyer, Folch, and Other methods are as follows: 1.43 ± 0.10, 2.67 ± 0.13, 1.85 ± 0.13, 1.02 ± 0.05, respectively. Different letters above the boxes represent a statistically significant difference at $p < 0.05$ (Tukey HSD).

provide a high estimate of total fibre that exceeds total carbohydrate content as measured by common total carbohydrate methods (e.g., Anthrone and Dubois methods), therefore we have pooled the total carbohydrate and fibre methods together for our analyses of carbohydrate content as percent dry weight (Fig. 3; Table 4). The estimate of carbohydrate content as percent dry weight (Table 3) is significantly different for all the methods examined (Tukey HSD, $p < 0.05$) except the By Difference and Prosky methods (Tukey HSD, $p = 0.105$). The By difference and Prosky methods are associated with the highest and similar median carbohydrate contents as percent dry weight and are significantly higher (Tukey HSD, $p < 0.05$) than those of the Anthrone and Crude Fibre methods, which produced the lowest median carbohydrate estimates.

We tested for the impact of drying method on carbohydrate content as percent dry weight determined using the Dubois method and a combination of the By Difference and Prosky methods. The Dubois method was the most commonly used total carbohydrate method ($n = 541$), and the By Difference and Prosky methods were combined because they provided the highest estimates of carbohydrate as percent dry weight and do not significantly differ from one another. For the Dubois method, drying at room temperature is associated with much higher estimates of carbohydrate as percent dry weight compared to all other methods (Tukey HSD,

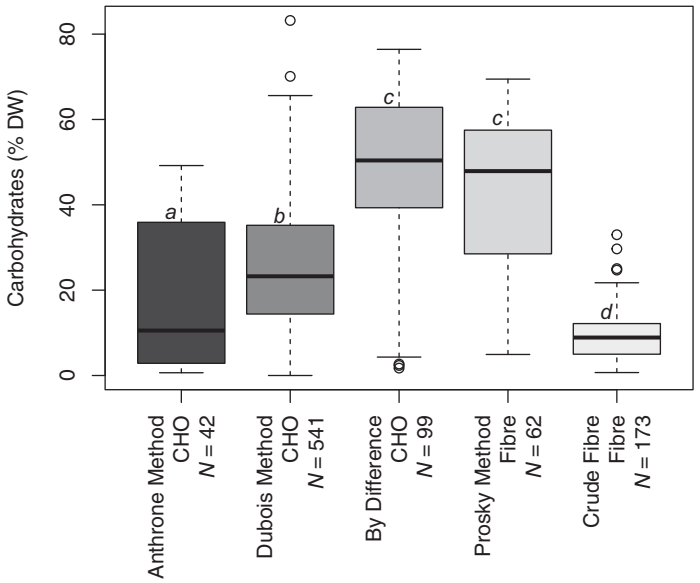

Fig. 3. Estimates of total carbohydrate (Anthrone, Dubois, By difference) and fibre (Prosky, Crude) as percent dry weight (% DW) as a function of method shown as box plots to illustrate the distribution of observations. Medians and standard errors of the methods from left to right are: 10.53 ± 7.16, 23.25 ± 0.66, 50.40 ± 2.67, 47.90 ± 2.85, and 9.00 ± 0.50, respectively. Different letters above the boxes represent a statistically significant difference at $p < 0.05$ (Tukey HSD).

Table 3. Median lipid content as percent dry weight extracted using the Bligh and Dyer and Folch methods as a function of drying method. Different letters represent statistically significant differences across methods at $p < 0.05$ (Tukey HSD). *N* is sample size. SE is one standard error. There were no observations for Bligh and Dyer using Sun-Dried or Blotted drying.

	Bligh and Dyer		Folch	
Drying method	**Median±SE**	***N***	**Median±SE**	***N***
Freeze-dried	2.90 ± 0.28^{a}	58	2.77 ± 0.21^{a}	48
Oven-dried	2.70 ± 0.18^{a}	70	1.90 ± 0.21^{a}	163
Room temperature	1.09 ± 0.73^{a}	45	$1.80 \pm 0.22^{a,b}$	24
Sun-dried	NA	0	$2.27 \pm 0.34^{a,b}$	3
Blotted	NA	0	$1.15 \pm 0.23^{a,b}$	42

$p < 0.001$, Table 4). For the Prosky/By Difference methods, oven-drying is associated with significantly lower estimates of carbohydrate as a percent of dry weight than freeze-drying (Tukey HSD, $p < 0.001$), but there are no other significant differences across the other methods examined (Tukey HSD, $p > 0.05$). All effects observed using the grouped data on carbohydrate content were also visible at the phylum level, so they are not reported here (nested ANOVAs and Tukey HSD tests).

Table 4. Influence of drying method on carbohydrate content as percent dry weight as determined by the Dubois or combination of Prosky and By Difference methods. See text for reasons for combining the Prosky and By Difference data. Different letters represent statistically significant differences across drying methods at $p < 0.05$ (Tukey HSD). *N* is sample size. SE is one standard error.

	Dubois method		Prosky, by difference	
Drying method	**Median ± SE**	***N***	**Median ± SE**	***N***
Freeze-dried	21.4 ± 0.58^{a}	58	55.5 ± 2.49^{c}	27
Oven-dried	23.1 ± 0.34^{a}	366	40.8 ± 1.09^{d}	84
Room temperature	41.0 ± 0.65^{b}	40	$52.8 \pm 1.69^{c,d}$	61
Sun-dried	NA	0	$52.3^{c,d}$	1
Blotted	23.6 ± 0.48^{a}	41	$40.6 \pm 1.63^{c,d}$	2

Discussion

The macromolecular composition of macroalgae is used to assess their potential for aquaculture, biofuels, and other biotechnological applications (Lee 1977; Lobban and Harrison 1994; Fleurence 1999; Nayar and Bott 2014; FAO 2016). There are a large number of methods available for estimating the macromolecular composition of macroalgae, however these are not standardized and the biases for each method are poorly characterized. This complicates the comparison and pooling of macromolecular data across studies, and hampers efforts to leverage work in the literature in order to identify species and strains of macroalgae that may be especially high in protein, lipids, or carbohydrates. Here we compile 125 studies of macroalgal macromolecular composition from the literature and show that there are systematic biases in protein, lipid, and carbohydrate content as determined by the most commonly used methods. Considering the clear underlying differences in the chemical bases of each method, these biases are unsurprising. Below we highlight how method selection, including the methods chosen to dry samples, influences estimates of protein, lipid, and carbohydrate content in macroalgae and make recommendations for methods to use for the determination of macromolecular content in macroalgae.

Protein as a percent of dry weight estimated using the nitrogen content method is 1.35 times the protein content estimated using a peptide-detecting protein assay in macroalgae. The nitrogen content method assumes that protein is 16% nitrogen by mass and that all measured nitrogen is from protein, which typically results in over-estimates of the actual protein content. Plants, microalgae, and macroalgae can contain significant quantities of non-protein nitrogen (Conklin-Brittain et al. 1999; Barbarino and Lourenço 2005; Angell et al. 2016; Finkel et al. 2016). For example, 10% of the total nitrogen in the brown macroalga *Macrocystis*

pyrifera (Linnaeus, C.Agardh) is non-protein nitrogen stores (Zimmerman and Kremer 1986). Chlorophylls, nucleic acids, and inorganic nitrogen stores all contribute to non-protein nitrogen in algae (Dortch et al. 1984; Lourenço et al. 2002). Environmental conditions and the distribution of non-protein nitrogen and protein nitrogen in macroalgae are also known to be closely linked (Angell et al. 2014, 2015). It is probable that inorganic nitrogen pools are responsible for the bulk of non-protein nitrogen in macroalgae, as chlorophyll and nucleic acid content in most macroalgae tends to be less than 0.2% (Orduña-Rojas et al. 2002; Chakraborty and Santra 2008; Rohani-Ghadikolaei et al. 2012; Turan et al. 2015; Vilg et al. 2015) and 0.8% (Mumtaj 2015) of dry weight, respectively.

The median ratio of protein as determined by peptide-detecting assays to that estimated using nitrogen content varies across the three major macroalgae phyla (Table 1). For the Chlorophyta, protein estimated using the nitrogen content method is 1.34-fold higher than when using the peptide-detecting assays. This is similar to values obtained for the pooled data (1.35-fold difference). By contrast, there is no significant difference in protein estimated using the two methods for the Ochrophyta. For the Rhodophyta, protein estimated using the nitrogen content method results in a 2.4-fold higher estimate than when using the peptide-detecting assays. Angell et al. (2016) also observed a larger difference in protein content between the two protein quantification methods for the Rhodophyta. Some of the variability at the phylum level may reflect different extents of inorganic nitrogen storage, as Rhodophyta have been shown to have a higher nitrogen storage capacity than the other macroalgal phyla (Fujita 1985). Experiments with the Rhodophyte *Gracillaria tikvahiae* over a range of nitrogen conditions show that protein mass can be far less than 6.25-fold nitrogen mass (with factors as low as 3), while non-protein nitrogen-rich compounds (inorganic nitrogen, amino acids, phycoerythrin) can be very high (Ryther et al. 1981; Bird et al. 1982). Additionally, the primary light-harvesting component of Rhodophyta is the pigment-protein complex phycoerythrin, which can account for 5.8–21.7% of total protein (Bird et al. 1982). In contrast, chlorophyll *a* dominates the pigment component of the Chlorophyta and Ochrophyta. The abundant pigment component of phycoerythrin and phycoerythrobilin is much more nitrogen-rich (C : N = 8.25) than pigments such as chlorophyll *a* (C : N = 13.75), and could contribute to an even higher non-protein nitrogen-content in Rhodophyta. Another possibility is a bias in sampling species that are higher or lower in protein with only one of these methods. Indeed, for the Rhodophyta, species that are recognized as protein-rich, such as *Porphyra tenera* and *Palmaria palmata* (Fleurence 1999) were only measured using the nitrogen-content method in our database, which could have elevated the median protein in comparison to the protein assay methods.

Given the large variability in protein content as determined by the nitrogen content method and the clear confounding effect of non-protein nitrogen, we strongly recommend using peptide-detecting assays to estimate protein in macroalgae. This recommendation comes with the caveat that the most common peptide-detecting assays have a varying response among types and sources of protein (Berges et al. 1993). Additionally, direct comparisons of peptide-detecting assays have demonstrated less sensitivity and greater variability in response with the Bradford assay, therefore the Lowry or BCA assay are preferred among these methods (Berges et al. 1993). If the nitrogen content method is to be used, we recommend using the macroalgae-specific nitrogen-protein factors determined by Lourenço et al. (2002), who rigorously compared how these factors vary across macroalgal species and phyla. While peptide-detecting assays, particularly the Lowry and BCA, are recommended among the methods evaluated in this study, it should be noted that more laborious methods involving extraction and complete hydrolysis of all peptides, followed by the colorimetic or chromatographic analysis of total amino acids, should be considered the benchmark methods for total protein and would likely provide the most accurate results (Fountoulakis and Lahm 1998).

Differences in the common lipid extraction methods are responsible for a ~2-fold difference in lipid as percent dry weight in macroalgae (Fig. 2). The Folch and Bligh and Dyer methods provide higher extraction efficiencies relative to the AOAC and Other methods, which can likely be attributed to the extraction solvents used. Different solvents are better suited for particular lipids (Randall et al. 1991; Manirakiza et al. 2001). For instance, triacylglycerides (storage lipids) can be extracted with relatively non-polar solvents such as chloroform, but more polar lipids associated with membranes require solvents such as methanol to be fully extracted (Randall et al. 1991). The Folch and Bligh and Dyer methods both use a mixture of chloroform and methanol. The AOAC methods use diethyl ether and ethanol, and the Other methods generally used dichloromethane, ethyl ether, or petroleum ether, which appear less effective in extracting lipids than a combination of chloroform and methanol. The chloroform and methanol mixture combines a moderately polar solvent with another that is slightly more polar, which is well suited to extract the range of lipid polarities found in macroalgae (from non-polar triacylglycerols to polar phospholipids and pigments). The AOAC or Other methods rely on a very non-polar solvent such as diethyl ether and thus primarily extract non-polar triacylglycerols, and have poor extraction efficiency for the variety of polar lipids found in macroalgae. While the AOAC 925.32 method uses two solvents of differing polarities, it involves an initial acid hydrolysis that causes deacylation, which extracts fatty acids from the glycerol backbone and polar head groups from complete lipids. Additionally, the aqueous alcohol in

the AOAC 925.32 method is used to create a biphasic system, isolating non-lipid polar contaminants in the aqueous alcohol phase, rather than to enhance the extraction of total lipids from samples. Based on the systematically higher values of lipid as percent dry weight obtained using the Folch and Bligh and Dyer methods, we recommend these methods be used over the AOAC method and other single solvent methods.

Additionally, the Bligh and Dyer method has a significantly higher lipid yield than the Folch extraction method (1.44-fold larger). Higher lipid yields using the Bligh and Dyer extraction method compared to the Folch method were also observed in microalgae and yeast (Ryckebosch et al. 2012; Castanha et al. 2013). Iverson et al. (2001) found that Folch achieved superior lipid extraction compared to Bligh and Dyer in a variety of marine animal tissues that were greater than two percent lipid by weight (Iverson et al. 2001). Macroalgae in the current dataset have an average lipid content below two percent of dry weight. Higher lipid extraction by Folch in lipid-rich animal tissues was attributed to the greater proportion of solvent to sample mass and the relatively less polar conditions of the Folch extraction, as higher lipid contents are generally due to the presence of non-polar triacylglycerols. The protocol for the Bligh and Dyer lipid extraction method involves an initial homogenization with 1 : 2 chloroform:methanol then a rehomogenization after addition of more $CHCl_2$ and water, which the Folch method does not do. Additional disruption from homogenization may aid extraction from macroalgal tissue. Based on the higher lipid values obtained with Bligh and Dyer extraction we recommend the Bligh and Dyer method as a default over all other lipid extraction methods for macroalgae.

Estimates of carbohydrate content in macroalgae as percent dry weight vary up to ~5-fold across the common methods examined; the highest estimates are associated with the By Difference and Prosky methods, and the lowest are associated with the Crude Fibre and Anthrone methods. Our analyses compare methods considered to be measures of the total carbohydrate pool with methods that measure the fibre pool (a subset of the total carbohydrate pool that is not digested in the gut). We pool our analyses of estimates of fibre with estimates of the total carbohydrate pool because our results show that the Prosky fibre method provides a higher estimate of carbohydrate than methods (Anthrone and Dubois) that are typically used to estimate total carbohydrate (Fig. 3).

The large variability in the estimates of carbohydrate and fibre content reflects the differences between the chemical assays used and assumptions made by these different methods. The low carbohydrate content as percent dry weight determined using the Anthrone and Dubois methods is likely due to the effectiveness of the polysaccharide hydrolization step in the beginning of each procedure. The types of polysaccharides present, the strength of the acid, temperature, and duration of the hydrolysis step can alter final carbohydrate extraction yield (Pakulski and Benner 1992; Woldu and Tsigie 2015). Both the Anthrone and Dubois methods use sulphuric acid and heating to 100°C to hydrolyze polysaccharides to monomeric derivatives which are quantified by colorimetry (Yemm and Willis 1954; Dubois et al. 1956). The key difference between these two methods is that the Dubois method includes phenol in the hydrolysis step to produce furan derivatives that form a yellow complex (Dubois et al. 1956), while the Anthrone method includes an anthrone solution in the hydrolysis step to produce hydroxymethyl furfurals that form a green complex in the presence of anthrone (Yemm and Willis 1954). The hydrolysis steps in these methods may not completely hydrolyze some more resistant macroalgal polysaccharides due to the concentration of sulphuric acid used or the duration of heating, although the addition of phenol in the Dubois method likely enhances extraction, since phenol denatures other cell components, such as protein. A modified Dubois method procedure with a more effective hydrolysis step may be a better method for chemically determining total carbohydrate content in macroalgae. Taylor (1995) found increased yield with the Dubois method when the concentration of sulphuric acid was increased, but still found very low assay response for recalcitrant polysaccharides such as cellulose and pectin (Taylor 1995). It should also be noted that if acid hydrolysis is too harsh, it can break down monomer sugar derivatives and reduce the apparent yields of some major sugars (Chow and Landhäusser 2004; Woldu and Tsigie 2015).

As non-specific colorimetric assays, both the Anthrone and Dubois methods include the underlying assumption that all carbohydrate monomers present after hydrolysis produce the same colorimetric response as the reference standard used. Glucose is typically used as a standard in these two methods, and both methods have been shown to have a large variation in colorimetric response between glucose and other sugars (e.g., xylose, galactose) (Yemm and Willis 1954; Dubois et al. 1956) that are common components of macroalgal polysaccharides. Taylor (1995) also found a low response in the Dubois method for some sugar monomers (rhamnose, *d*-galacturonic acid) that are often major components of complex algal cell wall polysaccharides. This differential response in both assays can cause an underestimate of total carbohydrate content depending on the carbohydrate composition of a particular sample.

As with the Anthrone and Dubois methods, incomplete hydrolysis of samples using the Crude Fibre method is likely the cause of its low carbohydrate yield. The Crude Fibre method partially digests samples with dilute sulfuric acid followed by dilute sodium hydroxide (Henneberg and Stohmann 1859) to mimic the respective conditions of the stomach and small intestine. This weak acid-base treatment is known to be insufficient to hydrolyze the recalcitrant

structural polysaccharides in plant material and thus has long been considered an inaccurate estimate of total or dietary fibre content (Mehta and Kaur 1992; Knudsen 2001). Other evaluations of weak acid hydrolysis of algal material have shown that these conditions are far less effective at hydrolyzing structural materials like cellulose, chitin, alginic acid, and oligosaccharides compared to concentrated sulphuric acid (Pakulski and Benner 1992).

The Prosky (fibre) and By Difference (total carbohydrate) methods provide the highest and arguably the best estimates of carbohydrate as percent dry weight in macroalgae with regards to the common methods assessed in the current study. However, neither of these methods provides a chemical measure of total carbohydrate content. Instead, they measure dry weight and subtract an estimate of protein and ash content and assume the remaining mass is carbohydrate. Lipids are typically not subtracted because they usually account for less than ten percent of dry weight in macroalgae (Prosky et al. 1987). The only difference between the Prosky and By Difference methods is that the Prosky method also removes starches and monosugars from measured dry weight. Other components unaccounted for include DNA, RNA, and pigments, which typically account for less than 5% of dry weight (Orduña-Rojas et al. 2002; McDermid and Stuercke 2003; Hong et al. 2007; Rao et al. 2007; Ganesan et al. 2014; Vilg et al. 2015). The small (not significant) difference in median carbohydrate content as percent dry weight estimated from the Prosky and By Difference methods (Fig. 3) suggests that the majority of the material left after the removal of protein, lipids, and ash, is fibre (Benjama and Masniyom 2011, 2012). That both the Prosky and By Difference methods provide higher values than the total carbohydrate methods is likely not due to their accuracy, but rather the ineffectiveness of the Anthrone and Dubois methods with macroalgal tissues. The Prosky method likely accounts for a lot of complex polysaccharides that are not necessarily fibre (McCleary et al. 2012), and the By Difference method is likely an overestimate of carbohydrate content since it does not directly measure carbohydrate. A recent method to estimate total dietary fibre is described by McCleary et al. (2012) and should be considered over the Prosky and crude fibre methods.

The drying method used in the preparation of samples before analysis can affect macromolecular yields. This is likely due to the degradation of specific macromolecules, altering the extractability of the macromolecules, or creating interferences that alter the detection of the macromolecules. Typically, macroalgae are dried in an oven or freeze-dried, but in some cases samples are dried at room temperature, in the sun, or simply blotted before analyses. There is evidence that following freeze-drying and oven-drying, approximately 10% moisture can remain in samples (Wong and Cheung 2001; Marinho-Soriano et al. 2007; Khan and Qari 2012; Rodrigues et al. 2015). Inconsistent and inefficient drying will result in higher water content and lower and more variable estimates of macromolecular content as a percent of dry weight. Surprisingly, blotting provided similar estimates of carbohydrate as oven drying using the Prosky and By Difference methods, and similar values of protein estimated from $N \times 6.25$ as other drying methods. In contrast, differences in temperature and humidity levels across labs is a significant source of variability in macromolecular content as percent dry weight in microalgae (Laurens et al. 2012). Our analyses indicate that drying macroalgal samples at room temperature provides the highest estimates of protein content as percent dry weight for the peptide detecting assays and the highest estimates of carbohydrate as percent dry weight for the Dubois, Prosky and By Difference methods as compared to all other drying methods. Oven-drying and freeze-drying may degrade carbohydrates. Smith (1973) found that drying using high temperatures (oven-drying) can cause plant carbohydrates to degrade and that after freeze-drying, some enzymes can still remain active. High temperatures can also reduce the extractability of proteins, reducing estimates of soluble protein content (Zweifel et al. 2003). It is possible blotting samples dry does not damage macromolecules or reduce their extractability, partially explaining why samples that were blotted dry had similar or better yields than other drying methods. In contrast to protein and carbohydrate, lipid as percent dry weight is higher (but not significantly) when samples are freeze-dried compared to all other drying methods. Lipids are easily oxidized under atmospheric conditions and freeze-drying is often recommended for sample preparation prior to the analysis of lipids and fatty acids (Halvorsen and Blomhoff 2011).

Our results are based on our analyses of methodological differences in estimates of macromolecular content across a large number of studies. The large sample size used in this analysis was advantageous, but some of the variability attributed to methodological biases could be due to different combinations of species with inherently high or low macromolecular content, differences in the environmental conditions under which the macroalgae were sampled, and idiosyncratic differences in the application of methods across individual labs. Therefore, further studies are needed to confirm these methodological biases in individual species, especially for carbohydrate, as was done for protein by Lourenço et al (2002), Barbarino and Lourenço (2005), and Angell et al. (2016) and lipid by Kumari et al. (2011).

Conclusions

There are numerous methods available to estimate the macromolecular composition of macroalgae. The most common methods tend to be those that are the easiest to use and require a minimum amount of specialized equipment and reagents. Although more complex and newer methods may provide improved extraction and detection efficiencies,

or quantify more specific subclasses of the macromolecular pools, there is a need for a standardized suite of methods that are well-known and easy to employ. These methods could be used by a wider community of researchers and would facilitate comparisons of the major macromolecular pools across studies.

Among those evaluated in this study, the most accurate methods for determining the macromolecular composition of macroalgae are the Lowry assay for estimates of protein, the Bligh and Dyer lipid extraction and gravimetric analysis for estimates of lipid, and the Prosky method for fibre and By Difference method to estimate total carbohydrate in macroalgae. A modified version of the phenol-sulfuric method (Dubois) or other carbohydrate assays with a more efficient hydrolysis step should be developed to directly measure total carbohydrate content in macroalgae. Drying at room temperature is recommended for measuring protein and carbohydrate content, and freeze-drying is recommended for determining lipid content.

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Acknowledgments

This work was supported by a Macromolecular Models of Marine Microbes grant from the Gordon and Betty Moore Foundation ID#3778 (ZVF, JL), the New Brunswick Innovation Foundation RAI program (ZVF, CF), NSERC Canada (AJI, ZVF), and the Canada Research Chairs Program (ZVF).

Conflict of Interest

None declared.

Submitted 13 December 2016
Revised 05 February 2017; 03 April 2017
Accepted 13 April 2017

Associate editor: David Suggett