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A data-driven comparison of commercially available testing methods for algae characterization

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ABSTRACT

Algal biomass composition is often the primary driver of economic viability of research and commercial algae-based product development. As the commercial algae production field is expanding, there is a growing need for a standardized language across producers and markets in terms of biomass characterization. Similarly, to establish and design collaborative or cross-institutional algae research projects, groups need to harmonize reported compositional analysis data to ensure comparable and reproducible results. In order to provide a consistent foundation of algae quality assessment, we are working towards closely coordinated, harmonized and objective operational protocols across the algae community. We present here a demonstration of biomass composition data from different commercial analytical laboratories, specifically applied to a reference biomass material. The data highlights a lack of consistency across laboratories in methods that are available for analysis. The data presented here illustrate variability in reported biomass measurements of protein, lipids and carbohydrate content causing differences of up to 1.36, 1.43, and 3.37-fold respectively, when expressed on a dry biomass basis. When the same methods are used between the laboratories, e.g. for protein and ash measurements, the data are consistent across laboratories. However, the reported lipid content varies with the method chosen at each laboratory, and causes the calculated carbohydrate content to then absorb the remaining difference from full mass balance closure. A suggestion of a common set of analytical standard methods available commercially is provided and can help to alleviate some of the challenges the algae community faces in terms of biomass descriptive reporting. This work sets the stage for and identifies critical niches to build on an established framework for understanding and harmonizing analytical methodologies for compositional analysis of algal biomass.

1. Introduction

In the nascent algae industry, progress towards feasible algae-based products depends on accurate biomass characterization, even though compositional analysis of algae throughout the literature has been reported with a variety of methods [1–8]. Strain selection and tailored growth conditions towards the effective production of high-value products can be economically advantageous. The methods needed to produce accurate and informative compositional data are not tailored to algal biomass and are often taken from a standard suite of methods in routine use at contract laboratories and thus can cause confusion when comparing processes, species or materials produced.

Current commercial testing methods are available for either nutritional, energy, or product testing, but there are no recommendations on which methods are applicable for algal biomass as a feedstock or sample. None of the existing available standards methods were specifically developed for, or tested on, algae. The methods are also not comprehensive enough to characterize the entirety of the biomass. This body of work concentrates on the available (primarily nutritional) testing methods, which are influenced by food labeling regulations and focus on nutritional approximates rather than summative mass balance accounting or a bottom-up approach. This key distinction between nutritional estimates and summative mass balance is the central issue with commercial testing on algae and is a limiting factor for process optimization and industry growth.

The difficulty with finding accurate and comparable analytical methods that are not just applicable but also tailored to algae is a challenge that has been described before [9–11]. The Algae Biomass Organization (ABO) in collaboration with the National Renewable Energy Laboratory (NREL) conducted an industry survey specifically tailored to poll respondents on the need and interest in a common language to help not just with harmonizing the biomass descriptive

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parameters but ultimately set the stage for quality parameter alignment. The survey was completed by a group made up equally of research scientists and industry representatives. There are two key points this survey highlighted: i) the algae industry is currently driven by overarching aquaculture and food and feed regulations and ii) there is a lack of available (custom) analytical methodology from contract labs. This survey confirmed that the industry was struggling with these issues and suggested that a lack of consistent and well-understood analytical methodology may hinder the algae market growth. Biomass composition is directly related to valorization, so variable results between different compositional analyses testing the same biochemical component can significantly influence value estimation [12–14]. Additionally, analytical results that are possibly biased based on the overall biochemical composition of the algae [10], would result in adjustments to cultivation operations that may not be economically beneficial.

When reviewing the methods that are available at contract laboratories, it was noted that they were not specifically developed for or validated on algae, and with no applicable algae standard reference material to check method validity, may not provide the information that is actually being sought by customers/industry. The standard methods most often used by commercial labs are maintained by the Association of Official Analytical Collaboration, most commonly referred to as AOAC International. However, none of these methods are currently validated for algae-specific products. While these methods may be mandated for the determination of certain components in food labeling, their use in other areas of the algae industry as well as for food labeling of algae products, without validation on algae, may lead to questionable results.

In contrast to summative mass balance, where components are added as they are identified, nutritional food labeling takes on a top down approach to compositional accounting, where 100% mass balance is assumed, and components are subtracted. This key distinction is mandated in U.S. nutritional labeling by title 21 of the Code of Federal Regulations (CFR 21) [15]. This code was established by the U.S. Food and Drug Administration (FDA) and covers a broad spectrum of food regulations in the U.S. The formula defining the mass balance of food products is shown below. According to CFR 21, carbohydrates in this equation are defined as follows, "Total carbohydrate content shall be calculated by subtraction of the sum of the crude protein, total fat, moisture, and ash from the total weight of the food" [15]. Therefore, carbohydrates are assumed to make up the difference between 100% and the other defined and measured components.

$$W_{sample} = W_{carbohydrates} + W_{moisture} + W_{protein} + W_{total\ fat} + W_{ash}$$

By this approach, the carbohydrate content is calculated and not directly measured, and therefore is particularly vulnerable to variation and uncertainty in the other measurements. In addition, it may not be an accurate reflection of the carbohydrate content if the other methods have not been validated on algae biomass. The assays used to identify everything except carbohydrates provide a method-based definition to rely on, while the top-down approach to calculating carbohydrates provides room to absorb the uncertainty, allowing the mandate to cover all food products sold in the U.S. This definition serves its purpose for food labeling but may not be applicable for all algae products. While algal biomass is similar to various food matrices, the food labeling methodology aims, above all, to be consistent across multiple different materials and matrices and does not aim to provide a full accounting of the biomass mass balance. Another difference between applications of methods to food stuff is the quantification of protein, which uses a standard 6.25 nitrogen-to-protein conversion factor. This factor is assumed for all food stuff, irrespective of the specific protein to non-proteinaceous nitrogen ratios of a given material. While this is not an accurate reflection of the true protein content of most materials, it is consistent across all nutritional labeling. If the conversion factor is not applicable to algae products [16], it results in an erroneous result for protein content and by extension, following CFR 21 guidance, also in reported carbohydrates.

The goals of the work described here were to i) identify a suite of standard laboratory analytical procedures that are routinely available at multiple commercial analytical laboratories and that provide standard information on the biomass composition, ii) characterize a reference algal biomass material representing two different species *Scenedesmus* and *Nannochloropsis*, and iii) compare the data obtained from commercial nutritional testing (top-down) to our in-house data, collected from a suite of analytical methods that take a bottom-up or summative approach to mass balance and that have been validated on algae biomass.

2. Materials and methods

The first stage of this research involved an exploratory investigation, which established a baseline of commercially available testing and eligible labs. From this investigation, three separate laboratories were chosen for sample analysis. These labs will remain anonymous in this publication, however some details on their spectrum of operation will be shared. One of the labs selected is currently one of the largest international commercial testing operations, with multiple testing facilities across the globe and specialty testing offered at many locations. Another lab is a subsidiary of a large oil and gas company, specializing in oil characteristics but also proximate testing, and had advertised specialty testing for algae biofuels. Lastly, the remaining lab was recommended to us by colleagues. This lab was a single facility operation who was known to have handled algal samples before, however this lab did not specialize in algae but rather proximate testing across a number of different applications. All three labs were ISO 17025 accredited. It is beyond the scope of this work to validate specific methods performed by these labs, however ISO 17025 accreditation indicates the labs ability to perform accurate analytical testing and frequent internal and external validation. ISO accreditation is issued by third parties to labs and covers competencies such as: equipment traceability, calibration, precision, quality assurance and technical competence. All methods used by the commercial labs were official AOAC methods, not proprietary methods developed by the labs, ensuring consistency in sample treatment and the reporting of results. A complete list of methods used can be found in Table 1.

Table 1
Summary of standard analytical methods available for the determination of the primary biochemical constituents in algae.

Standard method	Constituent	Method abbreviated summary
AOAC 945.46	Ash	Sample is combusted in 550 °C furnace until ash is carbon-free
AOAC 923.03	Ash	Sample is combusted in 550 °C furnace until light gray ash results or to constant weight
AOAC 986.25	Carbohydrates	Total Carbohydrates = (Total Weight) − (Protein + Fat + Moisture + Ash) - same as CFR title 21 [15]
AOAC 932.06	Fat/Lipids	Fat is extracted from sample after NH_4OH pretreatment with petroleum ether and ethanol in a 60–70 °C water bath
AOAC 954.02	Fat/Lipids	Fat is extracted from sample after HCl pretreatment with petroleum ether and ethyl ether in a 70–80 °C water bath
AOAC 996.06	Fat/Lipids	Fat is extracted with ether, then methylated to fatty acid methyl esters (FAMEs), followed by quantification via gas chromatography and reported as triglycerides
AOAC 992.15	Protein	Protein is calculated from measured nitrogen content by combustion, via 6.25 factor multiplication
AOAC 934.06	Moisture	Sample is weighed, dried for 6 h at 70 °C under vacuum in oven and then weighed again after reaching room temperature
AOAC 990.20	Moisture	Sample is weighed then dried at 100 °C for 4 h in an oven and then weighed again

Two samples representing two different species of reference material, consisting of 50 g of freeze-dried algal biomass each, initially characterized in our laboratory, were sent to each of the selected commercial laboratories. The species were *Nannochloropsis salina* and *Scenedesmus acutus LRB-AP-0401* (both originated at Arizona Center for Algae Technology and Innovation, AzCATI, at Arizona State University, Mesa, AZ). The *Nannochloropsis* was pond-cultivated under replete conditions in fall of 2014 in f/2 media in a 60 m^2 open pond in batch mode, while the *Scenedesmus*, also cultivated in outdoor ponds under fully nutrient repleted conditions, was harvested by centrifugation and lyophilized in 2016 and both biomass batches were kept frozen at −20 °C until 2018 shipment to NREL for packaging. The samples were separated from a 2–5 kg amount of algal biomass representative of a high protein/high nutrient harvest that was provided by Dr. John McGowen (Arizona Center for Algae Technology and Innovation, AzCATI, Arizona State University, Mesa, AZ). At NREL, the biomass samples were homogenized by mortar and pestle and sectioned into roughly 25 g portions, which were vacuum sealed with oxygen indicators, and stored at −20 °C. The characterization statistics of the reference biomass material were established within our laboratory as described in brief below. This reference material is freely available through inquiry on the following website: https://www.nrel.gov/bioenergy/microalgae-analysis.html.

Vacuum sealed packages of the material were shipped to commercial labs in insulated boxes containing dry ice to prevent degradation and possible changes in composition. All samples were shipped overnight to the commercial testing facilities with the insulation and refrigeration deemed necessary for their final destination. Labs were notified of shipment to ensure no samples went without any form of refrigeration for a prolonged period of time. The labs were advised to keep the material frozen while stored.

The biomass was characterized at NREL using a suite of methods previously described and applied to algae [3,17–19]. In short, for moisture, an aliquot of the sample was dried at 40 °C for 2 days and a percent moisture was determined [17]. All subsequent values were determined on a moisture-free (dry weight) basis. Dry oxidation (ashing) was performed on the oven dried sample as per the following NREL laboratory analytical procedure (LAP) [17]. For carbohydrate content, a two-step sulfuric acid hydrolysis was used to hydrolyze the polymeric forms of carbohydrates in the biomass into monomeric subunits. The monomers were then quantified by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [19,20]. The HPAEC-PAD method used to quantify the monomers deviates from the published procedure as follows: after hydrolysis, samples were filtered through 0.2 μm nylon filters and analyzed on a Dionex ICS-5000+ (Thermo Scientific, USA), equipped with a PA-1 column (Dionex #035391) and guard cartridge (Dionex #043096) set to 35 °C. The column was washed at 1 mL/min for 10 min at 200 mM NaOH then equilibrated for 30 min at 14 mM NaOH, after which a 20 min isocratic run at 14 mM NaOH separated the carbohydrates, which were detected by a pulsed amperometric detector (PAD). The lipid content as fatty acid methyl esters (FAME) was determined using the method described in the LAP referenced here [18]. In brief, 7 to 10 mg of biomass or the equivalent extract was dried for 2 days at 40 °C under vacuum before a dry weight was recorded. The dry samples were then homogenized with 0.2 mL of chloroform:methanol (2:1, v/v), and the resulting mixture was transesterified in-situ with 0.3 mL of HCl:methanol (5%, v/v) for 1 h at 85 °C in the presence of a known amount of tridecanoic acid (C13) methyl ester as a quantitative recovery standard. FAMEs were extracted with 1 mL hexane at room temperature for 1 h and analyzed by gas chromatography:flame ionization detection (GC:FID) on an Agilent 7890N; DB-WAX-MS column with dimensions 30 m × 0.25 mm i.d. and 0.25 μm film thickness. Details of the temperature program, flow rates, and standards were as described before [18,21]. Elemental nitrogen was determined by combustion with a CHN analyzer (Elementar Vario EL cube) following the Dumas method after which a conversion factor is used to calculate the protein content [22]. In this instance, the estimated protein content was calculated with a conversion factor of 4.78 [23]. To minimize error when determining the reference values, we followed ISO guide 35 and Institute for Reference Materials and Measurements (IRMM) guidelines for characterizing reference material.

A list of the most relevant commercially available analytical methods at commercial testing laboratories, is included in Table 1.

Samples were analyzed following acceptability criteria established at all laboratories, and data were guaranteed to fall within their respective laboratory quality control and regulatory acceptance criteria. As an example of a process of quality assurance and quality control (QA/QC), we document here an approach deployed at NREL to establish and implement a reference material for compositional analysis. The data included in Table 2 are reported as the running average of replicate datapoints for each analysis on the same reference biomass samples sent to the commercial labs, following statistical acceptance boundary descriptions that were calculated as follows. In brief, five replicates of the two reference material samples (taken from the same bottle) were analyzed by three different analysts on two different days, totaling 30 replicate measurements per analysis for each sample. Each analyst prepared their own calibration standards and curves from certified analytes. These initial data were used to calculate within bottle homogeneity of the two algae samples. Between bottle (vacuum sealed bag) homogeneity was determined by one analyst, for each measured constituent, analyzing 4 replicates from 10 separate vacuum sealed bags, totaling 40 data points per analysis. A one-way ANOVA performed on the between-bottle data indicated no significant differences. Data from both analyses were then pooled, for a total of 70 data points per analysis to determine the analysis precision and accuracy. Outliers present in the data were removed after checking for outliers using Tukey's fences (flagging data outside of the interquartile ranges).

3. Results & discussion

In order to establish a set of data based on commercially available analytical methods, a set of three laboratories were selected based on advertised specialties of analyses offered as well as whether they offered AOAC (or equivalent American Oil Chemists' Society, AOCS/American Society for Testing and Materials, ASTM) methods for the identification

Table 2
Measured composition and uncertainties for two algal biomass reference materials, after censoring data set based on Tukey outlier detection, u_{char} is uncertainty due to characterization expressed as the standard deviation, % relative standard deviation (RSD) is the percent relative standard deviation (stdev) and *N* is the sample size used to determine the average composition (as weight %) for each component measured.

Species	Analysis	wt% of biomass	stdev (u_{char})	% RSD	N
Nannochloropsis salina	Ash	17.8	0.20	1.13	68
	Carbs (PAD)	8.83	0.32	3.59	64
	FAME	9.68	0.17	1.80	70
	CHN_C	44.3	0.47	1.05	68
	CHN_N	6.69	0.08	1.21	68
	Protein (N)	31.98	0.39	1.21	68
	Amino Acid	31.07	n/a	n/a	n/a
Scenedesmus actutus	Ash	18.4	0.50	2.71	69
	Carbs (PAD)	9.43	0.30	3.17	67
	FAME	5.38	0.07	1.22	60
	CHN_C	43.6	0.43	0.98	58
	CHN_N	8.56	0.08	0.89	56
	Protein (N)	40.92	0.36	0.89	56
	Amino Acid	36.5	n/a	n/a	n/a

of protein, lipids, ash, moisture, and carbohydrates. After reviewing suggested methodologies and contacting the technical representatives, three labs were chosen, which will be referred to as laboratories A, B, and C in the remainder of this manuscript.

A summary of the standard analytical methods used by each of the three laboratories is shown in Table 3. Lab B did not perform the carbohydrate calculation, but this value was calculated based on the CFR 21 guidelines using the reported values for protein, lipid, ash and moisture. The method used by Lab C to estimate the carbohydrate content is equivalent to CFR 21.

The characterization of the two species (*Nannochloropsis* and *Scenedesmus*) reference biomass material with thorough statistical vetting and confirmation of homogeneity (as described in the QA/QC paragraphs above) made this material and its known compositional data a logical fit for this study into commercial compositional testing options.

Across commercial labs, the most consistent results were observed for the ash and protein components (Fig. 1). This was expected as all labs used similar methods to determine these two components (Table 3). The reported ash content is similar across all labs. The only difference in the method used by NREL to determine ash was a slightly higher final combustion temperature compared to the AOAC methods listed (575 °C instead of 550 °C). The relative standard deviation for the ash measurement based on an average and standard deviation determined from the data for all labs (including NREL) was 2.6% and 3.4% respectively for *Nannochloropsis* and *Scenedesmus*.

The protein values between NREL and the commercial labs appear different, but that difference is based on the nitrogen-to-protein conversion factor used, 4.78 for NREL and 6.25 for the commercial labs. If the effect of the protein conversion factor is removed, the relative standard deviation for the base N contents is 2.6% and 2.1% respectively for *Nannochloropsis* and *Scenedesmus*. The approach based on Kjeldahl nitrogen determination has been shown to give equivalent quantitative nitrogen data, following AOAC 954.01; AOAC 984.13; AOAC 988.05 [22]. The conversion factor of 6.25 is generally and globally accepted as a protein conversion factor, and is only appropriate for a narrow range of foodstuff, where the non-protein nitrogen content is minimal [24–26]. This is a consistent factor used in almost all food labeling, despite the fact that detailed analysis of either amino acid content and composition often yields a different calculated ratio. Specifically, characterizing the non-protein nitrogen components in algae has elucidated a value of 4.78 as most accurate for algae biomass, and is routinely used at NREL [16,23].

Variance in the results arise in reported fat (lipid) content. Labs A and B reported total lipid content as the measured fatty acid methyl esters (FAME). For Labs A and B, which both performed the same method, the lipid content for *Nannochloropsis* was 9.32% and 10.35%, and for *Scenedesmus* was 4.12% and 2.12%, respectively. Lab C reported the highest values for both species based on the gravimetric method, 14.01% and 6.99%. The higher fat content reported by Lab C is due to the method chosen and the resulting definition of fat rather than measurement inaccuracy. In that method, all extracted material is considered 'fat'. The differences in measured fat content, as well as the other components, carry through to the final calculated values for carbohydrates for each of the labs. For the lipid data, Lab C stands on its own due to the extraction method used and reporting only the gravimetric lipid yield. The AOAC 932.06 method uses an ammonium hydroxide pretreatment and petroleum ether extraction, followed by gravimetric determination of extractable lipids. Extraction methods often use different solvents and, because of lipid solubility differences, are not always specific for lipids, especially in a complex biochemical matrix like algae. It is possible that the higher reported fat content with the extraction-based methods is due to the coextraction of non-lipid compounds [10]. NREL and Labs A and B reported lipids based on the measured FAME content after transesterification and GC analysis, though based on different transesterification methods and different sets of quantified fatty acids. The relative standard deviation for the data from Labs A, B, and NREL combined was 5.3 and 43.6% respectively for *Nannochloropsis* and *Scenedesmus*. Inconsistency in reported fat values poses a challenge because it determines the potential for biofuel production. Non-lipid components co-extracted with lipids in gravimetric methods could lead to an overestimation of the potential for e.g. biofuel production.

Table 3
Summary of the AOAC methods used by each of the laboratories for the analysis of the reference material.

Component	Lab A	Lab B	Lab C
Ash	AOAC 923.03	AOAC 923.03	AOAC 945.46
Carbohydrates	CFR 21 calculated	Not provided	AOAC 986.25 calculated
Lipid	AOAC 996.06/ 954.02	AOAC 996.06/ 954.02	AOAC 932.06
Protein	AOAC 992.15	AOAC 992.15	AOAC 992.15
Moisture	AOAC 934.06	AOAC 934.06	AOAC 990.20

In our opinion, the most difficult data to compare are the carbohydrate data. The carbohydrate content measured by NREL, based on a direct detection method, was 8.32% and 9.13% for *Nannochloropsis* and *Scenedesmus* respectively. For the commercial labs, this component was calculated and absorbed differences in the other components as well as the fraction designated as unidentified in NREL's approach to mass balance. The results of the top-down approach are meant for nutritional labeling, to inform consumers of the nutritional composition in a consistent manner across foodstuff.

The contrasting approaches to mass balance, top-down and bottom-up, illustrate two very different ways of quantifying the biochemical composition of the algae samples used in this study. The most striking difference in the final compositional analysis summary is the inclusion of an unidentified component category in the NREL characterization, that is reflective of the summative (or bottom-up) mass balance approach used. The approach of only including identified and measured components for mass balance calculation makes for a careful accounting and unambiguous determination of the biomass composition. The remaining percentage of the mass is then labeled as unidentified, leaving room for the addition of other methods as determined by future research efforts. In the top-down approach, the unidentified fraction would be absorbed in the calculated carbohydrates. For large-scale algae biomass production and subsequent processing, detailed and accurate quantification of the components is crucial to the economics and performance metrics and may require a tailored set of methods or a distinct approach to algae biomass analysis.

4. Conclusion

Accurately identifying algal biomass components allows for industry expansion and acceleration of development of new products. While a suite of standard nutrional testing methods is available from commercial labs, they may not be applicable to characterizing algae feedstock. It is not always clear to the customer or consumer what each of the methods provide in terms of information that is valuable in the marketplace. We recognize that the datasets included in this work are not large enough for a comprehensive comparison of analytical methods against each other or against the NREL algae specific methods. We do however believe that the data set provided here is large enough to illustrate that the differences in analytical approaches are a concern for the growing algae industry and that the next steps in finding a solution to this would be a large scale study with established data bounds comparing different analytical methods and validating them on algae.

We urge the reader to use caution in the interpretation of compositional analysis data reported to the community, in particular algae carbohydrate data that may be calculated by subtraction. The herein reported characterization and respective biases in existing standard methods of a reference biomass material is a critical first step in defining

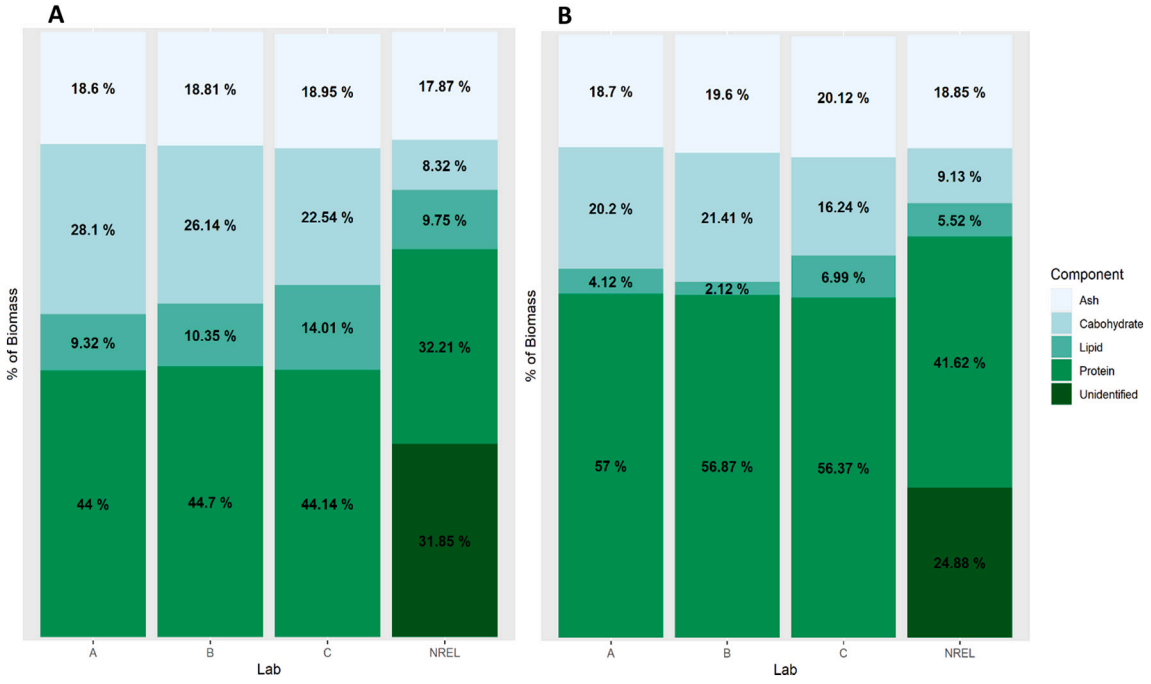

Fig. 1. Moisture corrected composition data (ash, lipids, carbohydrates, protein) for (A) *Nannochloropsis* and (B) *Scenedesmus* for Labs A, B, and C and NREL. Methods used for each laboratory are described in the text. NREL's values are an average of approximately 30 replicates per analysis, minus outliers.

an appropriate analytical approach for algae. Researchers are working to develop accurate standard analytical methods tailored to algal biomass and active efforts are ongoing to reach out to both commercial labs and industry members to start the conversation about standardization based on the needs of each sector. Efforts in this arena will help the industry move towards optimization and potentially diversify the application and commercial development space of algae.

CRediT authorship contribution statement

Lieve Laurens: Conceptualization, funding acquisition, methodology, writing, reviewing and editing, supervision; **Madeline Lane**: Data curation, analysis and original draft writing, reviewing and editing; **Stefanie Van Wychen**: Compositional analysis, data analysis, writing, reviewing and editing; **Andy Politis**: Statistics of internal composition data, data analysis and writing.

Declaration of competing interest

The authors declare no conflict of interest.

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Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

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