Suitability of Soxhlet Extraction to Quantify Microalgal Fatty Acids as Determined by Comparison with In-Situ Transesterification

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Abstract

To assess Soxhlet extraction as a method for quantifying fatty acids (FA) of microalgae, crude lipid, FA content from Soxhlet extracts and FA content from in-situ transesterification (ISTE) were compared. In most cases, gravimetric lipid content was considerably greater (up to 7-fold) than the FA content of the crude lipid extract. FA content from Soxhlet lipid extraction and ISTE were similar in 12/18 samples, whereas in 6/18 samples, total FA content from Soxhlet extraction was less than the ISTE procedure.

Abbreviations: FA (Fatty Acid), FAME (Fatty Acid Methyl Ester), TE (Transesterification), ISTE (*In-situ* transesterification), R-ISTE (Residual ISTE), TE-AH (TE preceded by Acid Hydrolysis), PUFA (Polyunsaturated Fatty Acid, GC (Gas Chromatography), TAG (Triacylglycerol), HPLC-CAD (High Performance Liquid Chromatography- Charged Aerosol Detection) LC-MS (Liquid Chromatography/Mass Spectrometry)

Re-extraction of residual biomass from Soxhlet extraction with ISTE liberated a quantity of FA equivalent to this discrepancy. Employing acid hydrolysis before Soxhlet extraction yielded FA content roughly equivalent to ISTE, indicating that acidic conditions of ISTE are responsible for this observed greater recovery of FA. While crude lipid derived from Soxhlet extraction was not a useful proxy for FA content for the species tested, it is effective in most strains at extracting total saponifiable lipid. Lipid class analysis showed the source of FA was primarily polar lipids in most samples (12/18 lipid extracts contained <5% TAG), even in cases where total FA content was high (>15%). This investigation confirms the usefulness of ISTE, reveals limitations of gravimetric methods for projecting biodiesel potential of microalgae, and reinforces the need for intelligent screening using both FA and lipid class analysis.

Key words: *In-situ* transesterification, Soxhlet, microalgae, biodiesel

Introduction

Microalgae have been suggested as a sustainable source of biodiesel based on a number of perceived advantages. Many species grow quickly, have the ability to accumulate a large proportion of triacylglycerols (TAG) as biomass under certain conditions, and can use waste sources of CO_2 and nutrients such as flue gas and municipal wastewater to support growth. The application of microalgae to economical production of biodiesel requires that a high percentage of microalgal biomass be composed of fatty-acid containing compounds that can be converted to fatty acid methyl

esters (FAME). This fraction, otherwise known as ‘saponifiable lipid’ includes neutral lipid such as triacylglycerols, but may also derive from other cellular components such as membrane lipids.

Considerable uncertainty remains as to whether it is possible to satisfy this requirement with scaled-up cultivation efforts. One of the challenges in resolving this question is the unclear meaning of various lipid content values reported in the scientific literature [1], generally referred to as ‘oil content’ or ‘lipid content’, with wide-ranging [2], and even unreferenced estimates commonly cited to make the case for microalgal biofuels [3]. In addition, there are researchers growing microbial heterotrophs [4] or algae heterotrophically [5], who consistently report higher lipid content values that may be included when modeling phototrophic biodiesel production as a sustainable fuel source. For example, the heterotrophic thraustochytrid *Schizochytrium* sp. and heterotrophic microalgae *Cryptothecodinium cohnii* were both included in a table that was labeled “Oil content of some microalgae” in a well-cited paper on microalgal biodiesel [3].

Contributing to this uncertainty, a number of different techniques are currently in use to evaluate crude lipid content, many based on the classic methods of Folch [6] and Bligh and Dyer [7]. Other examples include spectrofluorometric determinations where lipid bodies are directly stained and quantified [8], supercritical CO_2 extraction [9] and Soxhlet extraction [5, 10, 15]. It is clear that whichever method is chosen, careful attention is required to optimize the extraction conditions – pre-processing is in some cases essential [11], as is reaction time and reagent concentration [12, 13].

Even assuming optimized pre-treatment and extraction conditions, a key limitation of the above techniques is that they are unable to resolve lipid classes, as in

liquid chromatography or thin layer chromatography. Although it is apparent to experienced lipid chemists that lipid extracts may contain polar lipids, sterols, pigments, waxes, and oxidatively-altered fats in addition to triacylglycerols [14], many biofuel studies still rely on simple gravimetric estimations (hereafter referred to as 'crude lipid') to assess biodiesel yields [16, 17], despite the fact that including non-saponifiable lipid overestimates the suitability of a feedstock for biodiesel fuel. In contrast, other publications report crude lipid and FAME profiles [18], while others studies rigorously combine crude lipid, FAME profiles and quantification of FAME/TAG [19]. Also confounding simple comparison between studies is the polarity of the solvent used, which has a significant impact on the yield and composition of extracts from microalgae [15].

The use of gas-chromatography (GC) techniques, which yield accurate quantification and identification of FAME, is a simple procedure to resolve this uncertainty. FAME quantification gives information on the amount of biomass that is potentially convertible to biodiesel and also indirectly quantifies the amount of additional material extracted as crude lipid which has no value as a fuel.

The most commonly applied procedure for preparing FAME is an initial solvent extraction followed by transesterification (TE) of the resulting lipid with an alcohol, typically methanol [20]. To reduce the time required for this process, other authors [21] have suggested a single-step method where biomass is simultaneously extracted and transesterified, known variously as *in situ* transesterification (ISTE), direct transesterification, or one-step transesterification. Omitting the initial extraction step offers a significant savings in time and energy, results in increased precision [22], and offers a more complete extraction of fatty acids compared to conventional extraction (i.e.

Folch, Bligh & Dyer) followed by transesterification [21, 23]. The various applications of *in situ* transesterification have been thoroughly reviewed by Carrapiso and García [24].

In addition to requiring less time and potentially offering more complete extraction of fatty acids [21, 23], a key advantage of ISTE is that since the entire extraction/transesterification is carried out in a single vessel and quantified by GC, this procedure can be easily applied to small quantities (<10mg) of biomass without concerns for either loss of sample or analytical precision that would be expected using traditional extraction methods [24]. With these advantages in mind, a standardized ISTE protocol was developed in this laboratory, which was based on previously published standard transesterification procedures [25] as well as information from several recent investigations into ISTE of microalgal lipids [12, 13].

The goal of this investigation was to use ISTE to assess the relevance of gravimetric lipid data from Soxhlet solvent extraction to making projections about biodiesel productivity from microalgae. By comparing these two methods, it was possible to also investigate the factors responsible for the increased efficiency of ISTE procedures versus solvent extraction that have been previously reported [21]. Across 18 samples representing 16 species of microalgae, gravimetric lipid data was found to be an inconsistent metric for measuring total fatty acid content when compared with *in-situ* transesterification. This result confirms the effectiveness of ISTE for measuring total fatty acids in microalgae, and argues for caution in interpreting gravimetric lipid data from microalgae.

Experimental Procedure

Microalgal Culture

A list of the microalgae species cultivated to provide biomass for this study is listed in Table 1. All cultures were grown under continuous illumination, and all diatom cultures were supplemented with 100 μM silicate. Cultures were harvested by centrifugation with the exception of CCMP 1142, which was harvested by sieving. Photosynthetic batch cultivation was conducted in either enclosed 'Brite-Box' photobioreactors ranging in capacity from 200L-1000L [30] or in 18L plastic carboys. All batch cultures were harvested at stationary phase of growth. One strain, *Isochrysis galbana*, was cultivated in an N-limited chemostat and harvested at steady-state at a dilution rate of 0.5 d^{-1}. All cultures were lyophilized after harvest and stored at -20 °C prior to analysis. Three separate biomass samples of *Botryococcus braunii* Race B were analyzed, indicated by the numbers 1, 2 and 3.

Soxhlet Lipid Extraction

Freeze-dried biomass (0.5g) was homogenized with a mortar and pestle and extracted using the Soxtec 2050 automated solvent extraction system (FOSS North America, Eden Prairie MN) with the following program: boiling 25 min, rinsing 40 min, solvent recovery 15 min and pre-drying 2 minutes. Extraction temperatures for different

Table 1: Microalgae species used in the study, their taxonomic grouping and media requirements.

Species	Taxonomy	Media
Scenedesmus dimorphus	Freshwater chlorophyte	Freshwater with F/2 [26]
Porphyridium aerugineum UTEX 755	Brackish rhodophyte	10% *v/v* seawater/freshwater with F/2 media
Nannochloropsis granulata CCMP 529	Marine eustigmatophyte	Seawater with F/2
Nannochloropsis granulata CCMP 535	Marine eustigmatophyte	Seawater with F/2
Phaeodactylum tricornutum CCMP 1327	Marine bacillariophyte	Seawater with F/2
Neochloris oleoabundans UTEX 1185	Freshwater chlorophyte	Bold's 3N [27]
Botryococcus braunii Race B UTEX 572	Freshwater chlorophyte	Freshwater with F/2
Emiliania huxleyi CCMP 1324	Marine pyrmnesiophyte	Seawater with F/2
Tetraselmis chuii PLY429	Marine chlorophyte	Seawater with F/2
Glossomastix chrysoplasta CCMP 1537	Marine pinguiophyte	Seawater with F/2
Chlamydomonas sp. CCMP 2095	Marine chlorophyte	Prov 50 Medium [28]
CCMP 2321	Marine bacillariophyte	Seawater with F/2
CCMP 1142	Marine chlorophyte	Seawater with F/2
Chlorella vulgaris	Freshwater chlorophyte	Freshwater with F/2
CCMP 2327	Antarctic marine bacillariophyte	Seawater with F/2
Eutriptiella pomquetensis CCMP 1491	Marine euglenophyte	L1 Medium [29]

solvents were as follows: chloroform/methanol (2:1) - 150 °C, acetone - 150 °C, hexane - 155 °C, ethanol - 250 °C.

The solvents used in all Soxhlet extractions for two-step FAME analyses (TE) were chloroform/methanol (2:1). All solvents were HPLC grade or better (Fisher Optima). After extraction with the Soxtec 2050, lipids were dried at 105°C for 1 h, placed in a desiccator for 1 h, and weighed to determine gravimetric lipid yield. All values were standardized to ash-free dry weight of algal biomass unless otherwise indicated. Ash-free dry weights were determined by baking biomass overnight at 550 °C and reweighing the combusted residue [31].

Lipids were resolubilized in chloroform/methanol (2:1) and stored under nitrogen at -20°C until further analysis. Some extractions were done on residual 'defatted' biomass previously extracted in the Soxhlet apparatus (Fig. 1). Defatted biomass was kept in a fume hood to evaporate solvent, and stored at -20°C before re-analysis.

For acid hydrolysis pre-treatment, samples were processed with the Soxcap 2047 acid hydrolysis kit, according to application note 3907 (FOSS North America, Eden Prairie, MN). Briefly, the samples were boiled for 1 h in 3M HCl, rinsed with tap water until neutralized, dried at 60°C overnight and extracted in the Soxtec 2050 as previously described above.

Lipid Derivatization and *In-situ* Derivatization

Standard transesterification procedures [25] and recent investigations into ISTE of microalgal biomass [12][13] were used to develop lipid derivatization protocols. For two-step lipid derivatization (TE), a maximum of 20 mg of lipids extracted with the Soxtec apparatus were dried under nitrogen at room temperature to constant weight in pre-weighed pyrex tubes (previously baked at 450°C overnight to minimize lipid contamination). For *in situ* derivatization, a maximum of 20 mg of biomass was weighed directly into clean pyrex tubes. For both treatments, 1 mL of anhydrous toluene (Sigma-Aldrich) was added, followed by 2 mL of 5% acetyl chloride (Fluka)/anhydrous methanol (99.8% Sigma-Aldrich), prepared fresh for each reaction. Tubes were purged with nitrogen, capped, mixed gently, and kept at 105°C in a heating block for 1 h. Derivatized lipids were cooled to room temperature, washed with 5 mL of 18.2 MΩ H_2O with 5%

NaCl (w/v), 4 mL of 18.2MΩ H_2O with 2%$NaHCO_3$ (w/v), dried over anhydrous sodium sulfate and filtered through glass wool to exclude residual sodium sulfate.

Total derivatized lipid was then dried under nitrogen, weighed, diluted to 5 mg/mL with hexane and analyzed using gas chromatography. All reactions included a reagent blank to confirm clean solvents and glassware. Lipid and FAME yields were standardized to the ash-free dry weight of the biomass, with the FAME yield from the re-extracted defatted biomass represented as a percentage of the original biomass. Readers may refer to Fig 1 for a schematic description of these steps.

GC Quantification and Identification

All samples were analyzed on an Omegawax 250 column with an Agilent 7890 gas chromatograph equipped with a FID. Samples were run in constant flow mode (3 mL/min) and temperature programmed as follows: 110°C for 1 min, then 5°C/min to 250°C where the program was held for 20 min (total run time 49 min). Inlet temperature was set at 250°C, and detector temperature to 300°C. Carrier gas was helium. Peaks were automatically integrated by Chemstation software (Agilent).

A known quantity of internal standard (nonadecanoic acid, Sigma-Aldrich) was added to each tube before adding sample. The concentration of all other integrated peaks was calculated by calibrating to the area of the internal standard peak as per Tran *et al* [13] and Griffiths *et al* [21]. Individual fatty acids were provisionally identified by comparing retention times to two standard mixtures (Supelco 37 and PUFA No. 3, Sigma-Aldrich). Long-chain fatty acids (C28:1 and C28:2) in *B. braunii* were

provisionally identified from previous LC-MS analysis of Soxhlet lipid extracts (data not shown). For the comparison of individual FA, only components that comprised >2% of the total fatty acid pool by area % in the ISTE extracts were considered.

TAG Quantification by HPLC-CAD

Lipid profiling of the Soxhlet extracts of 18 different algal strains was carried out using HPLC with Charged Aerosol Detection (CAD). An Agilent 1100 HPLC binary pump was used with a Halo C8 50 x 3.0 mm column held at 40^{o}C. The detector was an ESA Biosciences Corona-Plus CAD detector operated with a N_2 gas pressure of 37 psi and the range was set to 100 pA. The mobile phase was delivered at 0.4 mL/min and consisted of A) 20% water / 80% methanol and B) 70% isopropanol / 30% methanol. The gradient was held at 50% B from 0 to 5 min, then increased from 50 to 100% B from 5 to 8 min, and held at 100% B from 8 to 15 min. The column was then equilibrated back to starting conditions for an 8 min period. Liquid chromatography solvents methanol and isopropanol were HPLC-grade and purchased from Caledon (Georgetown, ON, Canada). Samples were diluted to 0.1 mg/mL in methanol and filtered in 0.22 µm centrifugal tubes before analysis. Triolein (18:1/18:1/18:1) was purchased from Nu-Chek Prep (Elysian, MN, USA) and calibration standards ranged from 0.1 to 100 µg/mL. The injection volume in all cases was 10 µL.

Results

Figure 1 shows a flow diagram of the analyses that were performed for this investigation and the acronyms used to refer to them in the text. All microalgal biomass samples were initially extracted by both one-step ISTE and Soxhlet methods as described in Experimental Procedure. For the Soxhlet extractions, lipid extracts were subsequently transesterified to fatty acid methyl esters in a second step (TE). A number of additional processing reactions were then carried out on some of the 'defatted' biomass residues recovered from the Soxhlet apparatus to assess its effectiveness for determining saponifiable fatty acid content. Several residual biomass samples were subjected to re-extraction by the ISTE method (designated R-ISTE) in addition to back extraction by Soxhlet and transesterification (TE-2) in identical fashion to TE extractions. The residues from the TE-2 analysis were again subjected to ISTE analysis (designated R-ISTE-2). In other experiments, biomass samples were pre-treated with acid hydrolysis, subjected to Soxhlet extraction and transesterification (designated TE-AH).

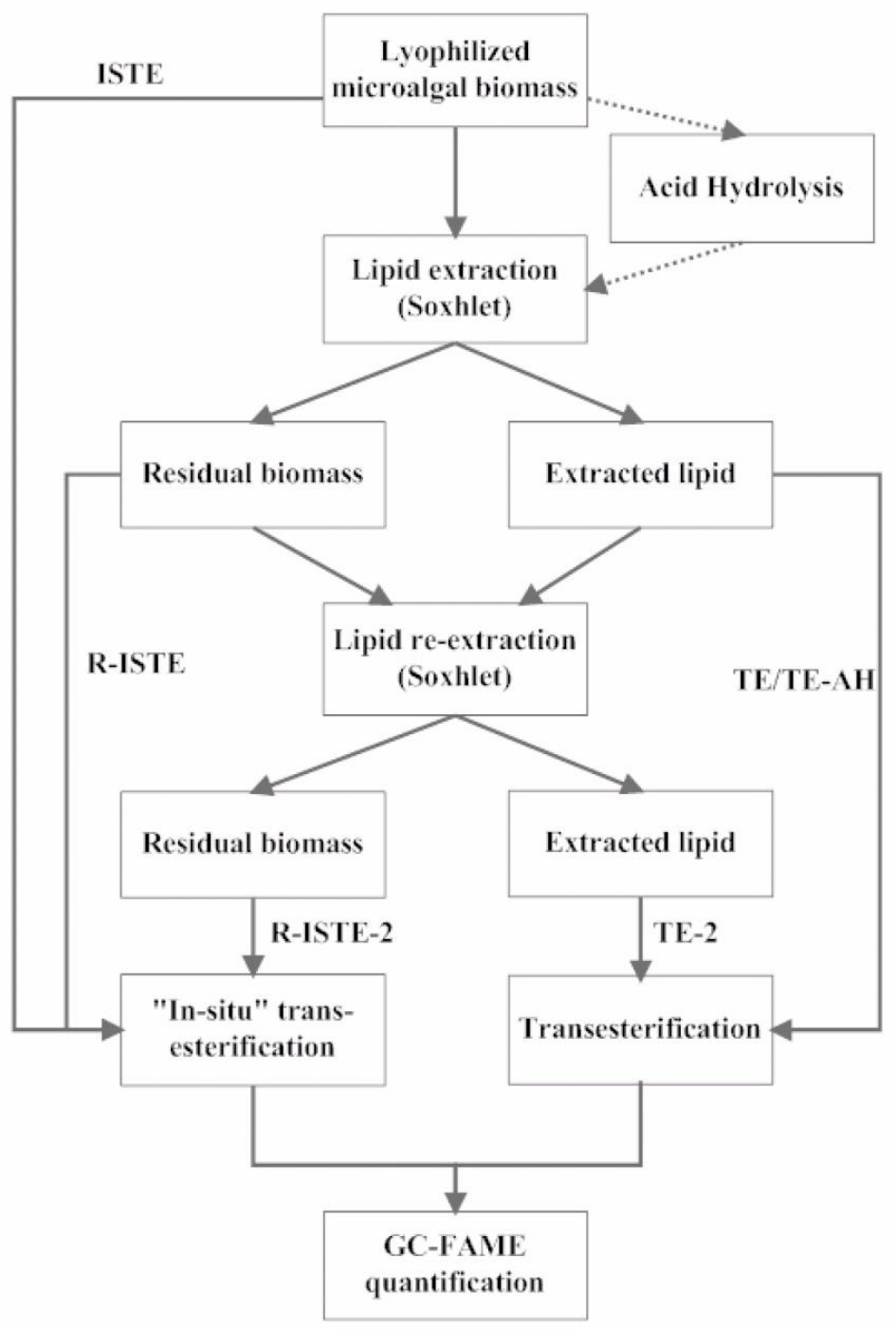


Figure 1 Flow chart describing lipid analysis as presented in Table 1, as well as Figs 2,3,4 and 5. The lipid re-extraction steps designated TE-2 and R-ISTE-2 are presented solely in Fig. 5. Acid hydrolysis pre-treatment followed by transesterification (TE-AH) was performed only for data presented in Fig. 6.

The Effect of Extraction Solvent on Gravimetric Lipid Content

Initial experiments on Soxhlet lipid extraction for three microalgal species (Figure 2) demonstrated a clear effect. Polar solvents (chloroform/methanol (2:1), ethanol and acetone) yielded higher lipid content, while the non-polar solvent hexane gave a lower yield. With the exception of *Botryococcus braunii*, which is known to contain a high proportion of neutral lipids such as TAG [32], hexane extracted little lipid (~4%) relative to more polar solvents such as ethanol or chloroform/methanol (2:1). Ethanol showed a higher variability in lipid content relative to the other solvent matrices.

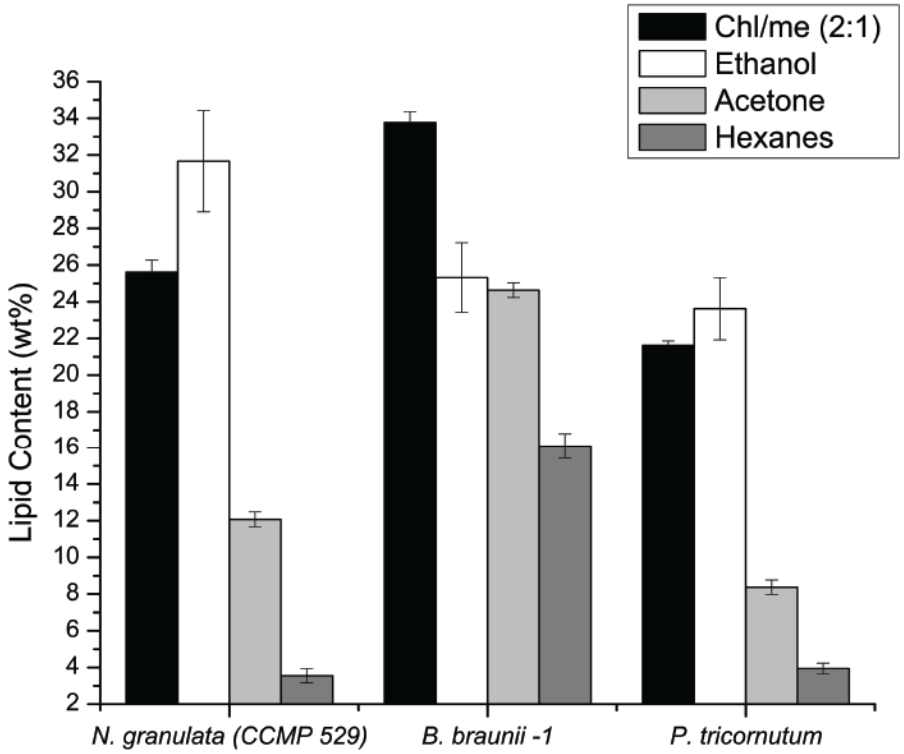


Figure 2 Lipid content using different solvent matrices for three microalgal species, as determined by Soxhlet extraction. Bars indicate standard deviation of the mean (n=6). Values are not standardized for ash or dry weight content of individual samples.

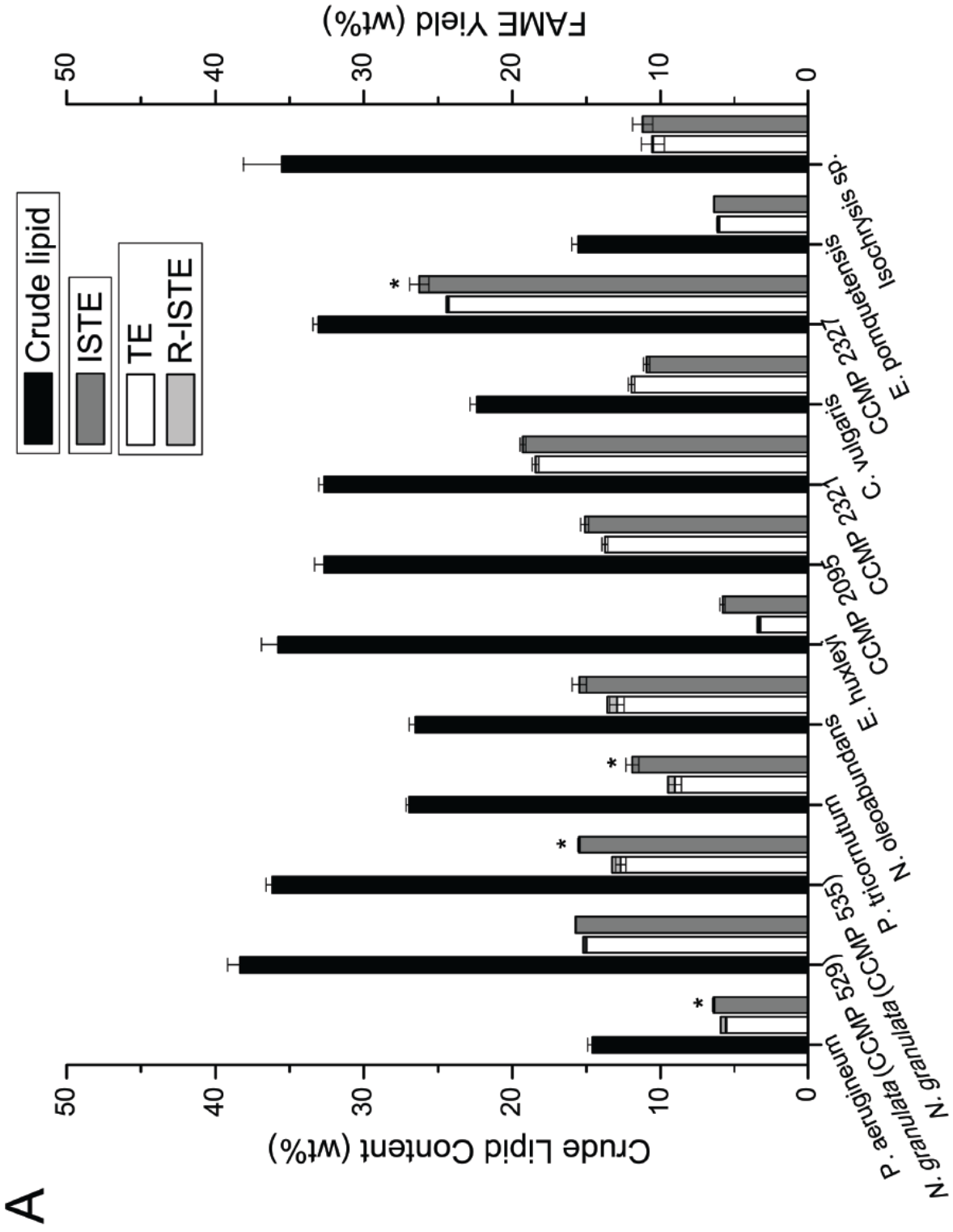
Comparison of Fatty Acid Content from Soxhlet and *In-situ* Transesterification (ISTE) Methods

Figure 3a shows that for 12 samples, TE and ISTE methods yielded a similar amount of FAME, with little or no FAME detected in R-ISTE extracts. In contrast, Fig. 3b shows that 6 samples yielded additional FAME with re-extraction of residual biomass from Soxhlet extraction (R-ISTE), indicating an incomplete extraction of fatty acids with the Soxhlet procedure. For *Botryococcus braunii*, the marine chlorophyte designated CCMP 1142 and *Tetraselmis chuii*, FAME yield derived from Soxhlet extraction-transesterification (TE) was not similar to *in situ* transesterification (ISTE). In two cases (CCMP 1142 and *B. braunii*-3), >50% of the total FAME extracted with the ISTE method was not detected with the TE method, but subsequently recovered with R-ISTE treatment.

Gravimetric Lipid Yield Compared with Total Fatty Acid Content

In 16 of 18 samples (Fig. 3a and 3b), gravimetric lipid content as determined by solvent extraction was, as expected, greater than the weight of fatty acids determined by quantification of FAME using both methods (TE, ISTE). In *Emiliania huxleyi* extracts, lipid content was ~7x greater than the fatty acid content as determined by TE or ISTE, but for most species the lipid content was between 2-3x greater than the total FAME

content. In two cases where Soxhlet extraction was not effective at liberating all



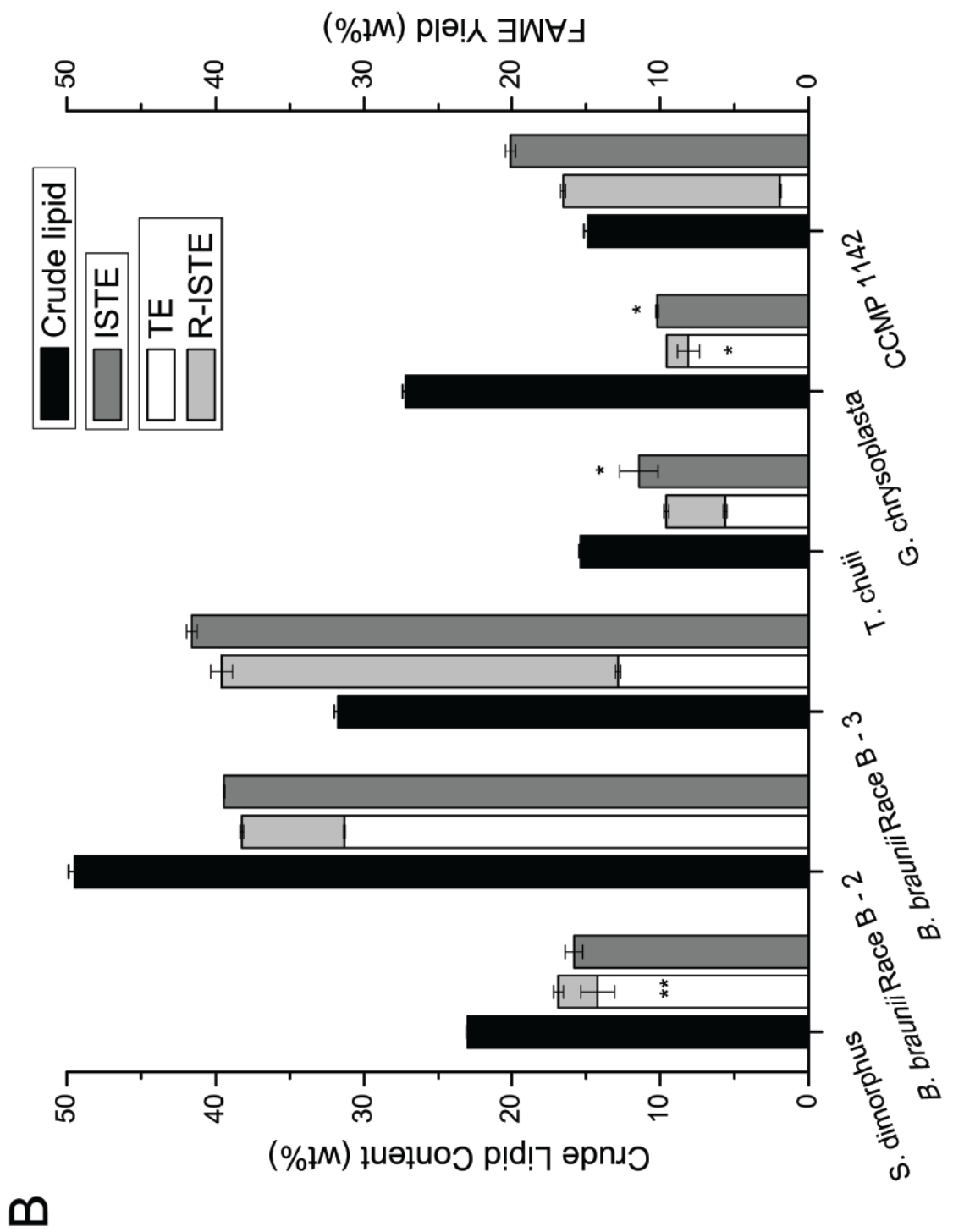


Figure 3(A&B) Comparison of gravimetric crude lipid content to FAME yield for microalgal biomass. Methods to determine yield of FAME include Soxhlet extraction followed by transesterification (TE), *in situ* transesterification (ISTE) and *in situ* transesterification of residual biomass from Soxhlet extraction (R-ISTE). Bars represent mean values with error plotted as range (n=2) except where otherwise indicated. Single asterisks (*) represent triplicates, and double asterisks (**) indicate n=4. In these cases, error is plotted as standard deviation of the mean. Values are standardized to AFDW.

extractable fatty acids (Figure 3b: CCMP 1142, *B. braunii*-3), gravimetric lipid content was less than the fatty acid content of the biomass as determined by ISTE.

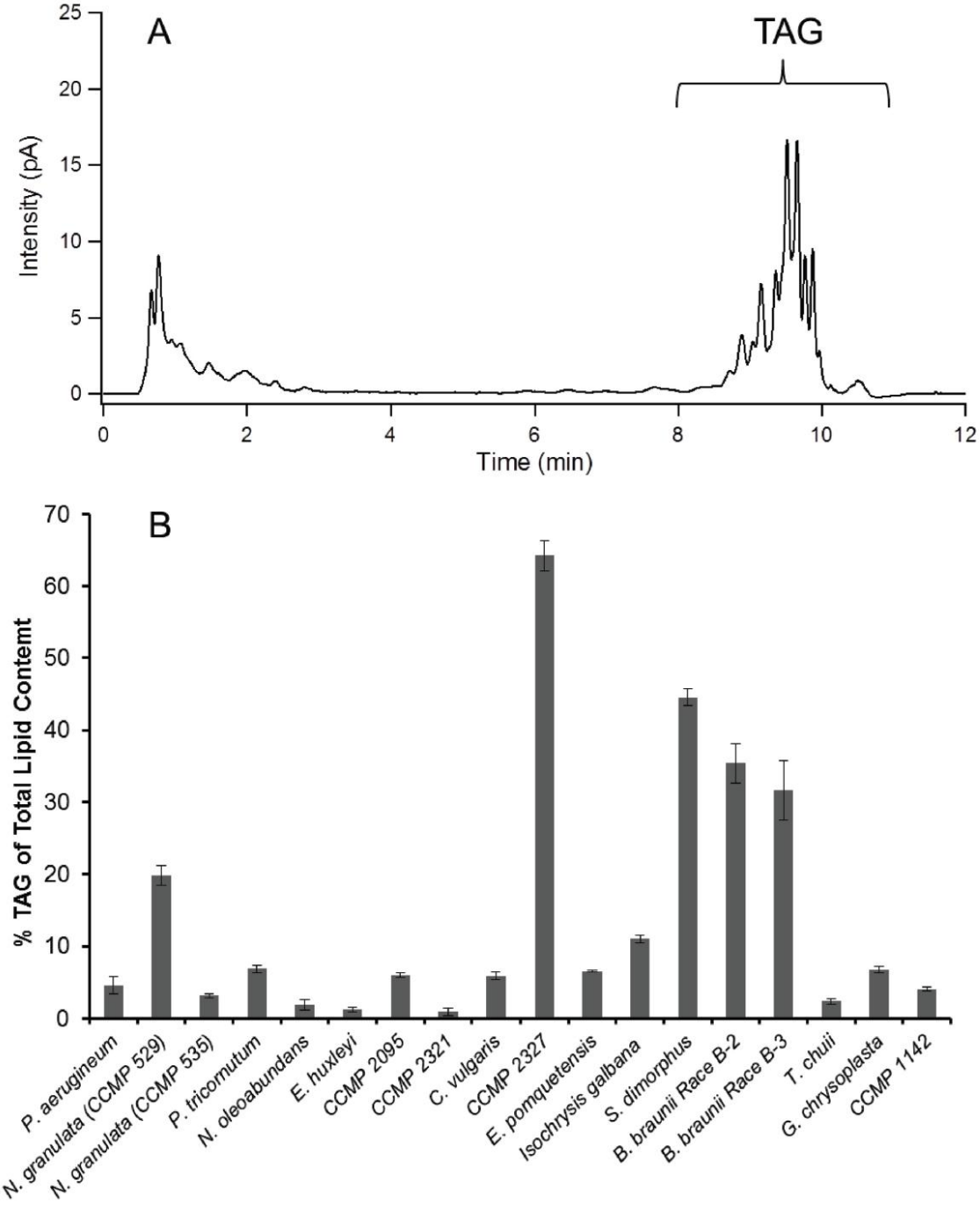


Figure 4 a) HPLC-CAD chromatogram of the Antarctic marine bacillariophyte CCMP 2337 and b) comparison of TAG content across the 18 algal strains, calculated as the percentage of the total lipid content measured gravimetrically using HPLC-CAD with triolein (C18:1) for calibration.

Intact Lipid Analysis by High Performance Liquid Chromatography

To gain an understanding of these apparent discrepancies between the gravimetric lipid content and the lipid content determined by GC-FAME analysis, 18 algal strains were also analyzed by HPLC-CAD to obtain profiles of intact lipids. Charged aerosol detection was chosen over the more commonly used evaporative light scattering detector (ELSD), as it offers higher sensitivity, wider dynamic range, and more consistent response factors [33]. The HPLC method was developed to rapidly resolve TAGs from other major lipid classes to allow for total TAG quantification, as shown in Figure 4a. Moderately polar lipids, such as free fatty acids, phospholipids, and chlorophylls eluted early in the chromatogram between 1-5 min, while TAGs eluted between 8 -12 min. TAGs were eluted over a narrow region of the chromatogram to minimize variation in response caused by the gradient [34]. Quantification of TAGs was achieved by summing the total peak area of the TAG region of the chromatogram and calibrating with triolein (C18:1), previously demonstrated as an effective calibrant for a wide range of TAGs [34]. Shown in Figure 4b are the TAG levels measured across the 18 algal strains, calculated as the percentage of the total lipid content measured gravimetrically. As anticipated, TAG content varied between species, ranging from roughly 1 % of lipid content for CCMP 2321 and *E. huxleyi* to as high as 64 % for the Antarctic marine bacillariophyte CCMP 2327. Futhermore, the strains with low TAG content exhibited high levels of the

Table 2: Extraction of specific fatty acids of using different techniques for 3 microalgal species. Values given as averages with ranges* (n=2) or averages with standard deviation† (n=3). Only individual FA comprising >2% of the area of total integrated peaks were used for this comparison. The values in column four are derived as indicated by the title. Percent in column 5 is calculated as follows: [R-ISTE/(R-ISTE+TE)] * 100%.

	mg FA/g AFDW				
Fatty acid	TE	R-ISTE	ISTE	Sum of R-ISTE & TE	% Sum represented by R-ISTE
a	*Botryococcus braunii* Race B				
	*	*	*		
C16:0	7.5 ± 0.0	3.9 ± 0.1	11.1 ± 0.1	11.3	34
C18:1n9	68.8 ± 0.5	193.9 ± 4.8	275.2 ± 3.3	262.7	74
C18:3n3	12.9 ± 0.0	5.8 ± 0.1	18.7 ± 0.1	18.6	31
C28:1	4.2 ± 0.0	21.9 ± 0.6	28.1 ± 0.4	26.1	84
C28:2	5.1 ± 0.0	29.7 ± 0.6	37.5 ± 0.3	34.8	85
Total FA	98.4 ± 0.6	255.1 ± 6.0	370.5 ± 4.1	353.5	72
b	*Tetraselmis chuii*				
	*	*	†		
C16:0	8.9 ± 0.1	7.8 ± 0.2	18.4 ± 0.4	16.7	46
C18:1n9	3.3 ± 0.0	2.9 ± 0.1	6.8 ± 0.2	6.1	47
C18:1n7	1.7 ± 0.0	1.4 ± 0.0	3.4 ± 0.1	3.1	46
C18:2n6c	2.7 ± 0.0	2.4 ± 0.1	5.7 ± 0.1	5.1	46
C18:3n3	7.7 ± 0.1	6.7 ± 0.2	16.1 ± 0.4	14.3	47
C18:4n3	7.6 ± 0.1	6.7 ± 0.2	16.1 ± 0.4	14.3	47
C20:5n3	3.2 ± 0.1	2.8 ± 0.1	6.8 ± 0.2	6.0	47
Total FA	35.0 ± 0.5	30.6 ± 0.8	73.3 ± 1.7	65.7	47
c	CCMP 1142				
	*	*	†		
C16:0	6.2 ± 0.0	28.4 ± 2.8	34.1 ± 0.3	34.6	82
C17:1	0.6 ± 0.0	5.0 ± 0.5	6.1 ± 0.1	5.6	90
C18:1n9	5.5 ± 0.1	30.1 ± 2.9	36.1 ± 0.4	35.6	85
C18:1n7	1.5 ± 0.0	7.6 ± 0.7	9.2 ± 0.1	9.2	83
C18:2n6c	3.9 ± 0.1	28.9 ± 2.9	34.8 ± 0.2	32.7	88
C18:3n3	2.9 ± 0.2	26.8 ± 2.6	32.4 ± 0.4	29.7	90
C18:4n3	0.7 ± 0.1	7.8 ± 0.7	9.6 ± 0.3	8.5	92
C20:1n9	0.6 ± 0.0	3.4 ± 0.3	4.0 ± 0.0	4.0	85
C22:6n3	0.8 ± 0.1	5.8 ± 0.5	6.7 ± 0.2	6.6	88
Total FA	22.6 ± 0.4	143.9 ± 13.8	173.1± 2.0	166.5	86

TE = 2-step transesterification (solvent extraction followed by acidic transesterification)
R-ISTE = 1-step transesterification of residual biomass from Soxhlet solvent extraction
ISTE = 1-step extraction-transesterification **FA** = Fatty acid **AFDW** = Ash-free dry weight

moderately polar lipids in the chromatograms, while those with high TAG levels yielded proportionally lower levels of the polar lipid classes.

Effect of Extractions on Recovery of Individual Fatty Acids

Table 2 shows that for all species tested, the sum of FAME from re-extraction of the residual biomass (R-ISTE) and Soxhlet extraction (TE) closely approximated the values for the initial *in-situ* procedure (ISTE), indicating that individual fatty acids were recovered effectively from residual biomass by the R-ISTE procedure. Table 2 also compares the amount of major fatty acids (>2% by weight) extracted with different procedures, standardized to original ash-free dry weight. For *Botryococcus braunii*-3 (Table 2), Soxhlet solvent extraction was selective in its effect, with C16:0/C18:3n3 being preferentially extracted with respect to other fatty acids. The marine chlorophyte CCMP 1142 showed a slight extraction bias for some fatty acids (e.g. C16:0 vs. C17:1), but *Tetraselmis chuii* did not follow a similar trend - all fatty acids were extracted with similar effectiveness.

Effect of Soxhlet Re-Extraction of Residual Biomass on the Extraction of Fatty Acids

In order to test whether or not the lower yield of fatty acids with Soxhlet extraction was due to non-optimized extraction conditions, pre-extracted (‘defatted’)

biomass was subjected to an additional Soxhlet extraction step. Fig. 5 shows that while

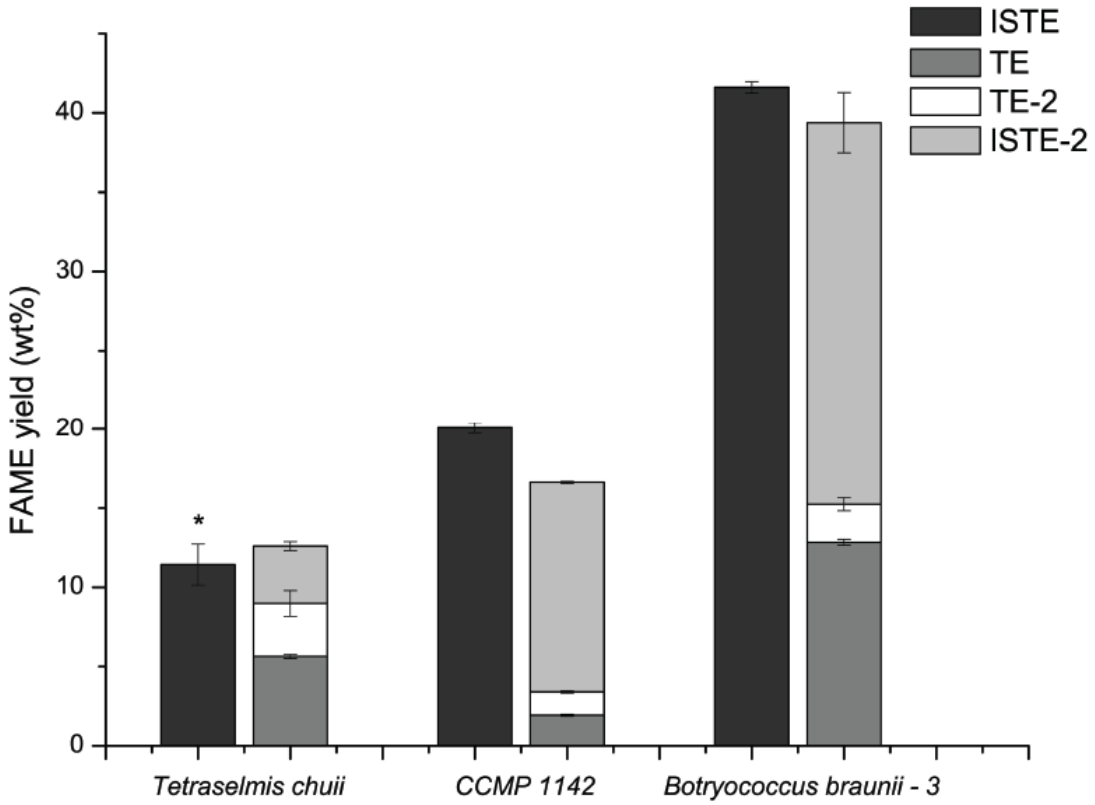


Figure 5 The effect of increased extraction steps on FAME yield for 3 species. Values obtained from *in situ* transesterification (ISTE) are plotted against the sum of transesterification of solvent-extracted lipid (TE), the subsequent re-extraction of 'defatted' biomass and transesterification (TE-2), and the *in situ* transesterification of the resultant biomass (ISTE-2). Bars represent mean values with error plotted as range (n=2) except where otherwise indicated. Single asterisks (*) represent triplicates, and in these cases, error is plotted as standard deviation of the mean. Values are standardized to AFDW.

additional extractions increased the FAME recovery in all species (TE-2), residual biomass from the second extraction still contained FAME recoverable by the ISTE method (R-ISTE-2). These results indicated that low fatty acid yields obtained in the initial Soxhlet extractions (TE) could not have been significantly improved by doubling the extraction time.

The Effect of Acid Hydrolysis on Soxhlet Extraction of Fatty Acids

A major difference between Soxhlet extraction and the ISTE procedure is the absence of an acidic catalyst with Soxhlet extraction. To test the importance of an acidic reagent for complete extraction of fatty acids, biomass was treated with an acid hydrolysis step prior to Soxhlet extraction and the results for four biomass samples are shown in Fig. 6. Acid treatment increased FAME yields in all strains tested, and in the case of *Botryococcus braunii*, fatty acid content with acid hydrolysis pre-treatment (TE-AH) closely matched ISTE values, in contrast to untreated biomass where there was a large discrepancy (Fig 3b).

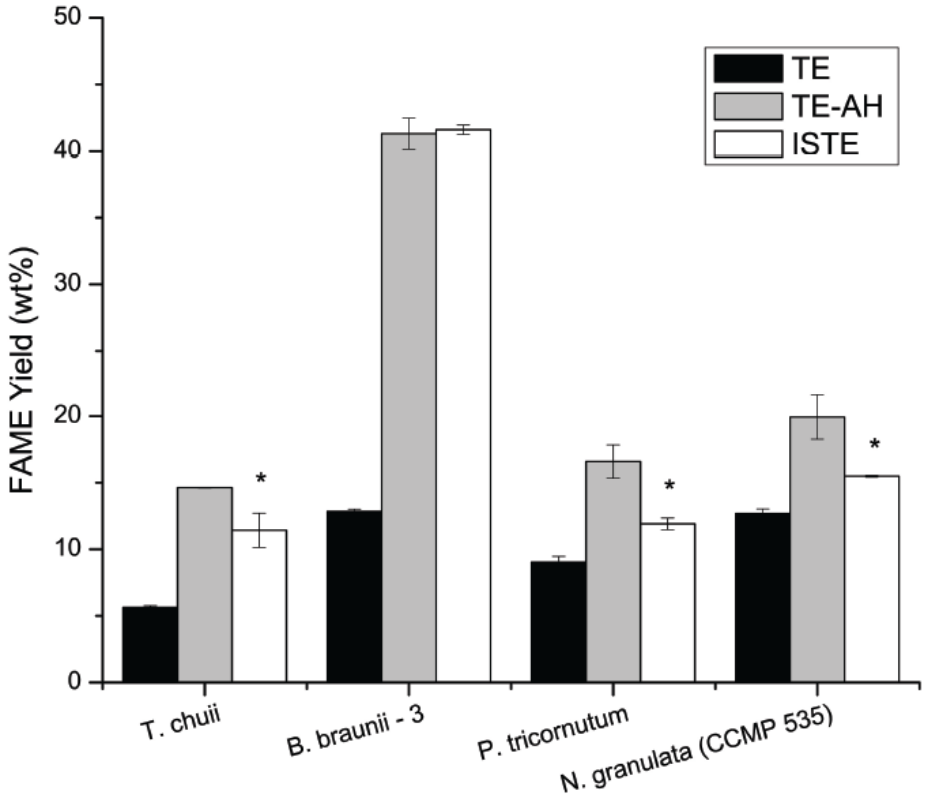


Figure 6 The effect of acid hydrolysis on FAME content of biomass. Solvent-extracted and transesterified lipid (TE) was compared to an identical extraction with acid hydrolysis pre-treatment (TE-AH) and *in situ* transesterification (ISTE). Bars represent mean values with error plotted as range (n=2) except where otherwise indicated. Single asterisks (*) represent triplicates, and in these cases, error is plotted as standard deviation of the mean. Values are standardized to AFDW.

Discussion

This paper investigated the effectiveness of using automated Soxhlet extraction to assess biodiesel potential of microalgal biomass. Soxhlet solvent extraction is a commonly used lipid extraction technique, and has the potential to be automated for high sample throughput. However, initial data generated in this lab from Soxhlet extraction of microalgal biomass showed that the choice of solvent had a large effect on gravimetric lipid content (Figure 2), which was anticipated and agrees with data from previous investigations [15]. For example, in the case of biomass from *Nannochloropsis granulata* (Figure 2, CCMP 529) hexane extracted ~3% lipid, while ethanol and chloroform/methanol (2:1) extracted >25% lipid on a dry weight basis.

Based on these higher yields with polar solvents, and observation of significant pigmentation in polar solvent extracts, it was hypothesized that polar solvents are able to extract additional polar lipids, as well as a significant quantity of non-esterifiable matter [14]. Any non-saponifiable lipid co-extracted with fatty acid-containing lipid will invalidate the use of gravimetric determinations from Soxhlet systems to estimate total fatty acid content.

In order to assess whether or not this was the case for several species, 18 samples of microalgal biomass were processed by Soxhlet extraction with chloroform/methanol (2:1), and subsequently transesterified with methanol to quantify FAME (Figure 3). In the Results section, the terms "FAME yield/FAME content" are used interchangeably as a proxy for total fatty acid content of biomass as determined by transesterification. Chloroform/methanol (2:1) was chosen for all analyses because of its ability to liberate

polar and non-polar lipids, as well as its wide application as a standard lipid extraction solvent in methods such as Folch and Bligh & Dyer.

In-situ transesterification was used as a comparative method to assess the efficacy of Soxhlet extraction based on previous reports indicating ISTE is more effective than standard solvent extraction methods at liberating fatty acids from biomass [21], as well as our experience that showed the identical phenomenon. Initial experiments with *Botryococcus braunii* biomass showed a discrepancy in the yield of FAMEs between the TE and ISTE methods, with TE giving considerably lower yields. Based on this data, residual biomass from Soxhlet extractions was analyzed with the ISTE method (R-ISTE) to attempt to explain this difference. These data, summarized in Figure 3, show that *in-situ* transesterification yielded comparable or higher values than extraction-transesterification (TE) in all but one case (*C. vulgaris*). While it appears that Soxhlet extraction was effective at liberating all fatty acid-containing lipid for the majority of species tested (Fig. 3a), ISTE was reliable even in cases where the Soxhlet system did not extract all saponifiable lipid, showing a similar result to a recent investigation [21] which compared ISTE to the lipid extraction techniques of Folch, Bligh & Dyer and Smedes & Askland.

A key finding of this study is that gravimetric yields were not a reliable estimate of total fatty acid content. While this result is not unexpected, we were able to show that the magnitude of this difference was highly variable between samples. Data presented in Figures 3a and 3b shows that in most cases gravimetric lipid content does not correlate well with total fatty acid content, with gravimetric lipid content being several-fold greater in many cases (Figs 3a and 3b), and in certain samples (Fig 3b) actually underestimating

total fatty acid content of the biomass due to incomplete Soxhlet extractions. Thus, Soxhlet methods used alone can significantly over/underestimate the potential of specific microalgal strains for biodiesel production, resulting in projected values that can be several times larger than values determined by quantification of FAME. In addition, variations in the percent of FAME in gravimetric extracts between samples (Fig 3a, 3b), precludes the use of gravimetric values as an estimate of fatty acid content across diverse strains.

The technique of *in-situ* transesterification proved to be a useful tool that did not share this weakness, and data on individual fatty acids extracted (Table 2) show that re-extraction of residual biomass (R-ISTE) accounted for the balance of individual fatty acids. This confirms that the ISTE procedure is extracting the balance of FAME that were not liberated by Soxhlet extraction rather than extracting additional lipid. Interestingly, values for polyunsaturated fatty acids corresponded well between the two treatments, indicating minimal oxidative damage from Soxhlet extraction, despite exposing extracted lipids to a drying step at 105˚C for 1 hour without protection from oxygen.

By comparing the ratio of fatty acids extracted in the TE and R-ISTE treatments to the total for these two treatments, it could be determined that for *Botryococcus braunii* (Table 2), Soxhlet extraction was selective in its effect, with C16:0/C18:3n3 being preferentially extracted with respect to other fatty acids, indicating that certain FAME-convertible lipids are more ‘recalcitrant’, notably C28:1, C28:2 and C18:1n9. Data from CCMP 1142 and *Tetraselmis chuii* did not follow a similar trend (Table 2) - all fatty acids were extracted with identical effectiveness. It should be noted that the results obtained from the *B. braunii* analysis indicate that either technique used in isolation (TE, ISTE)

will produce a different FA profile. This argues for caution when interpreting fatty acid data for microalgae, as the extraction technique chosen may bias the profile obtained [15].

In order to test the possibility that the lower FAME yields from TE were due to inadequate and therefore incomplete Soxhlet extractions, residual biomass from 'recalcitrant' strains was subjected to an additional, identical solvent extraction treatment, followed by TE of the lipid generated (TE-2) and ISTE of the resulting residual biomass (R-ISTE-2). The results (Figure 5) show that this additional treatment did not extract all of the remaining fatty acids, indicating that manipulating Soxhlet extraction conditions will not likely result in the maximum yield of fatty acids in this case. It should be noted that these 'recalcitrant' fatty acids in *B. braunii* have been shown to be present at high abundance as C28:1/C18:1/C18:1 and C28:2/C18:1/C18:1 TAGs by previous liquid chromatography-mass spectrometry (LC-MS) studies [35]. These TAG may be difficult to extract due to their large molecular weight, though further analysis of intact TAG would be required to confirm this. Alternatively, *B. braunii* is known to produce degradation-resistant biopolymers that include the identified C28:1/C28:2 fatty acids [36], which may also explain this phenomenon.

In order to understand why *in-situ* transesterification was able to more effectively extract total fatty acids, several experiments were conducted. The use of an identical solvent matrix as the ISTE procedure with the Soxhlet system did not result in an increase in total FA content (data not shown), so it was hypothesized that the acidic nature of the transesterification catalyst was responsible for this pattern. Acidic treatments have been demonstrated to increase the extraction of fatty acids from

microalgae [37], presumably due to the hydrolytic release of lipid from other macromolecules as in conventional acid extraction [24].

To demonstrate that acidic treatment was necessary for maximal FAME yields in certain samples, an acid-hydrolysis pre-treatment step was employed for 4 species (Fig. 6). There was a clear effect of acid hydrolysis, with total fatty acid yield being increased in all cases, although TE-AH approximated the ISTE procedure only in the case of *B. braunii*. This is likely due to the cumbersome nature of this procedure – the extracted lipid from this procedure is not easily re-solubilized in the extraction solvent, and sample loss during boiling steps is possible. In addition, a disadvantage of this procedure is that it yields a gravimetric lipid percentage that is in some cases up to twice that from non-hydrolyzed biomass (data not shown). In the case of *Botryococus braunii*, this resulted in yields of >70% lipid, which is unlikely to accurately reflect 'oil' or biofuel potential, unless other hydrocarbons known to accumulate in *B. braunii* under certain conditions [38, 39] are present in large quantities (not measured in this study).

In addition to offering more rigorous extraction conditions, acidic catalysts have the additional advantage of being able to transesterify all classes of saponifiable lipid [25], which will undoubtedly include compounds other than neutral lipids, such as phospholipids and sphingolipids. In this manner, this technique will likely give an upper limit for the maximum amount of biodiesel that can be produced from microalgal biomass, neither underestimating (Fig. 3b) nor overestimating in the case of crude lipid determinations (Fig 3a; [9, 40]).

While ISTE analysis is a useful tool, it gives no information on lipid class, a critical consideration for biodiesel where TAG is the preferred feedstock. Thus, while

ISTE can give information about esterifiable lipid content, one cannot determine the original source of the FAME. We therefore decided to use HPLC-CAD to quantify TAG and polar lipids as a percentage of the gravimetric extract. For several samples, the overestimations of fatty acid content by gravimetric yields inversely correlate with TAG levels as measured by HPLC. For instance, *E. huxleyi* exhibited the largest discrepancy between gravimetric lipid content and FAME content (Figure 3a), while TAG levels were measured at just greater than 1 % of overall lipid content (Figure 4b). In contrast, the Antarctic marine bacillariophyte CCMP 2337, *Scenedesmus dimorphus* and *B. braunii* Race B-2, which showed the smallest discrepancies between gravimetric lipid content and FAME content by ISTE, yielded the highest relative TAG contents of the lipid extract at 64, 45, and 35 %, respectively (Figure 4b). In contrast, the samples CCMP 2321 and *T. chuii* which also showed a low discrepancy between ISTE and gravimetric yield, had very low TAG content (<5%, Fig 4b), showing that while the extract had a high fraction of esterifiable matter, TAG was not the main source of the FAME detected. For samples CCMP 1142 and *B. braunii* Race B-3, Soxhlet extraction did not effectively extract total esterifiable lipid, so comparisons could not be confidently made.

Most of the remaining extracts examined that showed larger discrepancies between gravimetric lipid yield and FAME yield by ISTE had correspondingly lower TAG content, with the exception of *N. granulata* CCMP 529 which showed 20% TAG content of the lipid extract. Therefore, the presence of various moderately polar lipid classes and other co-extracted compounds that do not generate FAMEs upon esterification, contribute significantly to the overestimations of gravimetric yields for

total fatty acid content. Also, these data show that higher FAME content derived from ISTE does not necessarily suggest TAG accumulation (e.g. CCMP 2321, Fig 3a, Fig 4b).

In this investigation, the majority of microalgal strains analyzed do not contain greater than ~15% esterifiable lipid with low TAG content, a yield that is likely to decline in an industrial-scale extraction scenario. It is important to point out however, that with the exception of the single N-limited *Botyrococcus braunii* culture (Fig. 3b, *B.braunii* Race B-2), and the *I. galbana* chemostat culture (Fig 3a), none of the microalgae used in this study were cultivated under conditions designed to induce lipid accumulation. Under conditions of nutrient stress, many species of microalgae respond by accumulating lipid, particularly TAG, in storage bodies, presumably as a mechanism to dissipate excess energy capture and/or as a readily available source of energy to mobilize when favourable conditions return [1, 2]. The comparatively low FAME yields presented here may be due to the cultivation conditions employed, which were designed to maximize growth rate and biomass productivity, rather than lipid content.

This investigation has confirmed the potential of strains such as *Botryococcus braunii* for biodiesel production, but has also shown that under conditions of fast growth, other purportedly oleaginous strains have low FAME yields, suggesting minimal triacylglycerol accumulation. Lipid content values obtained from hexane extractions strongly suggested minimum neutral lipid content, a conclusion supported by LC-MS analysis [35]. In the future, our lab will be following the lead of other investigators [19, 42] to directly quantify TAG accumulation to support these analyses.

This study confirms the main advantages for using *in situ* transesterification which include increased precision and accuracy, reduced time and labour costs,

elimination of time-consuming pre-treatment [11] and easy applicability for routine screening of new microbial isolates [4]. In this lab, replicable FAME data from small scale algal physiological experiments is routinely generated from biomass samples of ~1mg, something not easily achievable to the authors' knowledge with two-step extraction-transesterification procedures. In addition, the previously mentioned strategies for increasing algal lipid productivity are poorly understood from a physiological perspective [2], and ISTE will be useful in determining whether or not increases in neutral lipid content represent an increase of the total cellular lipid content, rather than a reallocation of FA from one pool to another [41].

Results obtained here argue for caution in projecting oil productivities of microalgal culture using solely gravimetric analyses. The crude lipid values obtained in this study correspond well with the values cited by Chisti [3] for the 'oil content' of selected species of microalgae, including many of the species studied in this paper. Such gravimetric data might lead an inexperienced analyst to conclude a strain has a high potential for biodiesel production, despite the fact that gravimetric data alone cannot give an accurate measure of biodiesel potential.

While Soxhlet extraction of microalgae does not yield crude lipid values useful for judging biodiesel potential, it appears that it is an effective method for extracting total lipids for class analysis, based on the similar FAME yields between TE and ISTE procedures for 12/18 samples investigated here. Based on this data, we suggest the use of quick and accurate GC-FAME analyses such as ISTE, combined with lipid class analysis by GC-TAG or HPLC-CAD to generate an accurate value for 'biodiesel potential' of

microalgae. When standardized to daily productivity [43], this offers a robust technique to evaluate microalgal strains for the production of biodiesel.

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