Bioresource Technology 129 (2013) 51–57

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

A rapid and general method for measurement of protein in micro-algal biomass

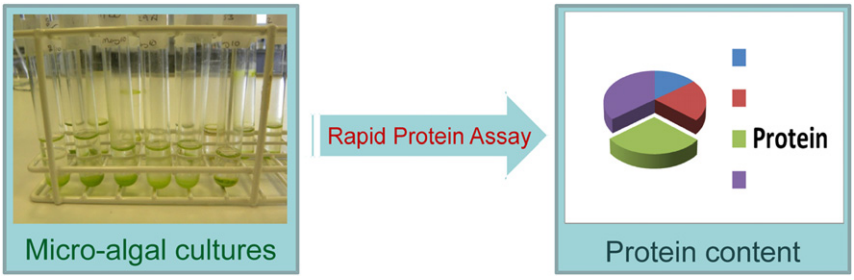
Stephen P. Slocombe *[,1], Michael Ross [1], Naomi Thomas, Sharon McNeill, Michele S. Stanley

Scottish Association for Marine Science, Scottish Marine Institute, Oban, Argyll PA37 1QA, UK

HIGHLIGHTS

- A rapid, small-scale method for micro-algal protein content.
- Optimized by testing with strains from diverse phylogenetic origins.
- Sequential hot-TCA and alkaline extraction can extract lyophilized material.
- Effective against recalcitrant *Chlorella* and Eustigmatophycean strains.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form 30 October 2012
Accepted 31 October 2012
Available online 10 November 2012

Keywords:
Intelligent screening
Protein extraction
Value-added products
Algal biomass
Aquaculture

ABSTRACT

A convenient small-scale extraction method for lyophilized micro-algae is described that dispenses with labor-intensive homogenization and is widely applicable to algae from different phyla. The procedure employs an optimized sequential extraction in trichloroacetic acid (TCA) and NaOH to achieve chemical lysis. Conditions were tested using several micro-algal strains to develop a method that was generally applicable. Incubation of lyophilized material in 24% (w/v) TCA at 95 °C followed by a hot alkaline treatment was found to be effective for strains that are resistant to conventional extraction approaches, such as the *Chlorella* and the Eustigmatophycean species. The single-tube extraction procedure can be complete in 4 h and is conveniently followed by the Lowry assay, requiring a further 30 min. Overall, this method proved to be generally applicable and ideal either for single samples or for high-throughput screening of multiple algal strains for protein content.

1. Introduction

Factors such as climate change, population growth, diminishing oil reserves and sustainability issues in the fishing industry have led to a resurgence of interest in micro-algae as a source of biomass, biofuels, fishmeal replacement and food (Becker, 2007; Day et al., 2012; Hu et al., 2008; Huntington, 2009; Stephens et al., 2010). Micro-algal protein in particular, has potential for animal feed or human consumption, recombinant protein technology and as a valuable by-product of biofuel production (Becker, 2007; Potvin and Zhang, 2010; Williams and Laurens, 2010). Marine strains could avoid conflicts with agriculture for freshwater supplies, e.g. *Nannochloropsis*, used both in fish-farm aqua-feed and large scale biofuel production (Day et al., 2012; Radakovits et al., 2012; Rodolfi et al., 2003).

Despite this potential, issues exist with the ease at which algal proteins can be extracted from some strains, such as *Chlorella*, due to their resilient cell walls (Doucha and Lívanský, 2008). In order to evaluate protein levels in novel strains and micro-algal collections, a rapid but generally applicable extraction procedure was needed. Lyophilized biomass was preferred as the starting material to avoid inaccuracies associated with measuring FW (fresh weight) in micro-algae due to liquid carry-over. Additionally, most other concomitant extraction procedures require dry biomass such as rapid direct-derivatization for fatty acid yield, or Dubois for carbohydrates (Carrapiso and García, 2000; Dubois et al., 1956).

Techniques that can be used to measure protein content rapidly in lyophilized material include Dumas-based combustive methods

* Corresponding author. Tel.: +44 1631 559353; fax: +44 1631 559001.
E-mail addresses: Stephen.Slocombe@sams.ac.uk (S.P. Slocombe), mikeyross@hotmail.com (M. Ross), Naomi.Thomas@sams.ac.uk (N. Thomas), Sharon.Mcneill@sams.ac.uk (S. McNeill), Michele.Stanley@sams.ac.uk (M.S. Stanley).
[1] These authors have equal contributions.

0960-8524
http://dx.doi.org/10.1016/j.biortech.2012.10.163

of elemental analysis and Kjeldahl to measure N-content (Fowden, 1954; Kjeldahl, 1883; Rebolloso-Fuentes et al., 2001). Protein can also be estimated from total amino acids by HPLC after hydrolysis of the dried material (Brown, 1991; Volkman et al., 1993). Nevertheless, in practice these approaches can lead to an overestimation due to a lack of specificity towards protein (Barbarino and Lourenço, 2005). Micro-algae often contain substantial levels of free amino acids and other N-organic compounds such as chlorophyll (Brown, 1991; López et al., 2010).

Dye-based procedures such as Bradford and Lowry largely overcome these problems and are more appropriate when applied to a wide variety of strains such as high-throughput screening procedures (Bradford, 1976; Lowry et al., 1951). Extraction procedures that incorporate TCA precipitation of protein can also remove TCA-soluble factors that may interfere with estimation (Clayton et al., 1988). Nevertheless, micro-algal extraction procedures for dye-based protein assays generally require prior extraction of protein by homogenization, which can add to processing time (Berges et al., 1993; Clayton et al., 1988; Conover, 1975; Rausch, 1981). Since lyophilized material is generally harder to extract protein from, most methods tend to start with fresh material (Walker, 2002). Recent procedures have been described for lyophilized micro-algae but also require labor intensive disruption steps either by pestle and mortar with inert ceramic particles (López et al., 2010) or use of the Potter's homogenizer (Barbarino and Lourenço, 2005). Furthermore, some commonly studied strains such as *Chlorella* or members of Eustigmatophyceae (e.g. *Nannochloropsis*) can present general extraction difficulties possibly due to small cell-size or resilient cell walls (Chiu et al., 2009; Doucha and Lívanský, 2008). These difficulties could be compounded when extracting dried material from these strains and this requires investigation.

In some reported procedures, it was evident that extraction at high temperature in alkaline solution (2 N NaOH at 95 °C; Pruvost et al., 2011) or hot-TCA (Price, 1965) could achieve chemical lysis without the need for homogenization but this was only shown for fresh material and with a limited number of strains. The method of Price (1965) employed very brief incubation in hot-TCA (6%, w/v) followed by extraction of the TCA pellet in 0.1 N NaOH (55 °C). This relatively mild treatment was sufficient for *Euglena gracilis* which is fragile and easy to extract protein from (Price, 1965) but not for other micro-algae (Rausch, 1981). Both steps were prolonged by Conover (1975) to extract from the diatom *Thalassiosira fluviatilis* but a homogenization step was also included. Conditions for extraction solely with alkaline solution were investigated by Rausch (1981), where short incubations at 80–100 °C, with concentrations up to 0.5 N NaOH were found to be optimal and could avoid losses by hydrolysis. It was concluded that this method gave higher yields than the TCA-based methods. Nevertheless, it was apparent from this earlier literature that the sequential hot-TCA- and alkaline solution extraction steps described in the original Price procedure could be developed into a widely applicable procedure for lyophilized material but this has never been investigated so far (Conover, 1975; Price, 1965; Rausch, 1981).

Therefore the aim of this study was to develop a rapid, small-scale and relatively simple protein extraction method suitable for single samples or high-throughput applications such as micro-algal screening. As such the desired method needed to be compliable over a broad taxonomical group and capable of dealing with lyophilized material. Furthermore, the treatment needed to be harsh enough to break down the strongest cell walls without homogenization but without compromising dye reactions through excessive degradation of the protein, once it had been released.

2. Methods

2.1. Micro-algal strains and culture

The seven species of micro-algae analyzed are listed in Table 1. All micro-algal species used were obtained from the Culture Collection of Algae and Protozoa (CCAP, UK). Starter cultures of 100 mL were incubated under a 12 h/12 h L/D (Light/Dark) regime at 50–80 $\mu E/m^2/sec$ at 20 °C for 7–10 d, without shaking (Innova 44, New Brunswick Scientific, Edison, NJ). Sub-samples from this were then inoculated into triplicate 500 mL aerated flasks containing 400 mL F/2 media using artificial seawater at 33.5 g/L (Instant Ocean, Aquarium Systems, France) (Guillard and Ryther, 1962). Each flask was exposed to 150 $\mu E/m^2/s$ light for 16 h/8 h L/D, at 20 °C throughout, in a controlled environment room, except for *Rhodella violaceae* which was exposed to 50 $\mu E/m^2/s$ light. Once the cultures reached stationary phase, they were harvested by centrifugation at 4000*g* for 15 min (Sigma 4K15 centrifuge, Buckinghamshire, UK). The harvested cells were then flash-frozen in liquid nitrogen, and freeze-dried for 3 d (ALPHA 1-2 LD plus freeze dryer, Christ, Osterode, Germany). The freeze-dried algae biomass was then transferred to individual glass vials, with a Teflon-lined stopper, and stored in the dark, under nitrogen gas at −80 °C.

2.2. Protein extraction

The small-scale method developed for protein extraction of micro-algal dry-weight (DW) was based on that used by Price (1965) with extensive modifications (Fig. 1). For each micro-algal species; 5 mg (±10%) of freeze-dried micro-algae material was weighed out. Three separate extractions were carried out for each experimental condition to determine variation in yield. Samples were resuspended by vortexing in either 250 μL 6% (w/v) TCA or 200 μL 24% (w/v) TCA. Homogenates were incubated in a water bath at either 65 °C or 95 °C, for 15 min, in screw-capped micro-centrifuge tubes and allowed to cool to RT. The samples containing 24% (w/v) TCA were diluted to 6% (w/v) with the addition of 600 μL ultrapure water. The homogenates were centrifuged at 15,000*g* for 20 min at 4 °C (Microcentrifuge 5415 R, Eppendorf AG, Hamburg,

Table 1
Micro-algal strains used in this work.

Class	Genus	Species	CCAP No.	Origin	
				Isolator (date)	Location
Chlorophyceae	*Dunaliella*	*primolecta*	11/34[a]	Gross (1936)	Marine; off Plymouth, Devon, England, UK
Prasinophyceae	*Tetraselmis* sp.		66/60	Keller (1982)	Marine; Oyster Pond, Falmouth, Massachusetts, USA
Trebouxiophyceae	*Chlorella*	*ovalis*	211/21A	Butcher (1953)	Brackish; River Crouch, Althorne, Essex, England, UK
		spaerckii	211/29A	Butcher	Marine; shellfish tanks, Conwy, Wales, UK
Eustigmatophyceae	*Monodopsis*	*subterraneae*	848/1	Lewin (1949)	Freshwater; rock surface, river at Marion, Connecticut, USA
	Nannochloropsis	*oculata*	849/1[a]	Droop (1953)	Marine; Skate Point, Isle of Cumbrae, Scotland, UK
Rhodophyceae	*Rhodella*	*violaceae*	1388/6	Eggert (2004)	Slightly brackish; Öland Island, Baltic Sea, Sweden

[a] Type culture.

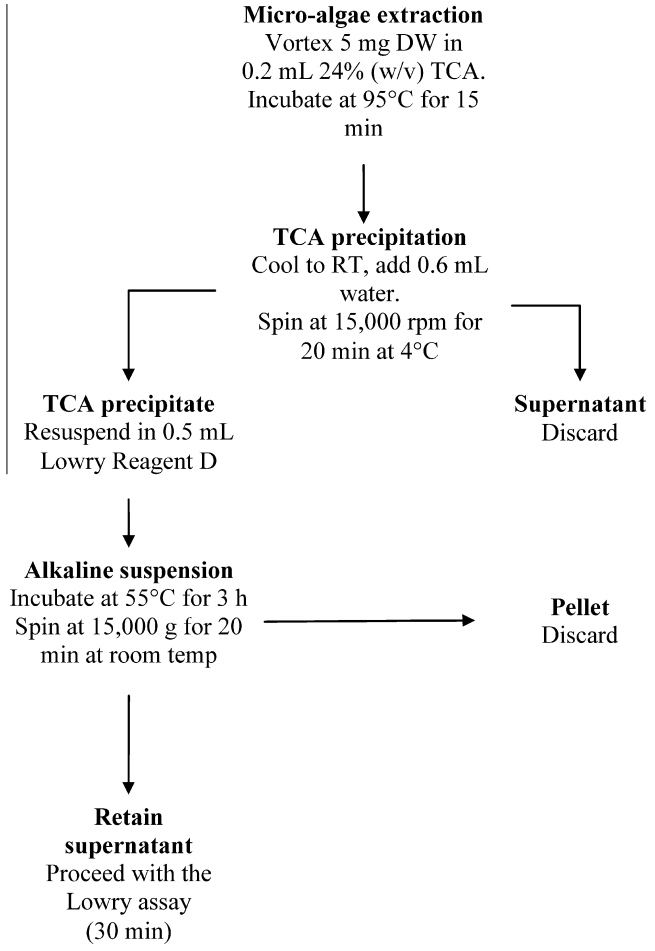


Fig. 1. A step-wise diagram of the optimal hot-TCA method for micro-algal protein extraction as proposed in this study.

Germany) and their supernatants discarded. The pellets were resuspended in 0.5 mL Lowry Reagent D (see Section 2.3) by repeated pipetting or vortexing and incubated over a series of time-points (10 min to 22 h) at 55 °C. Samples were then cooled to RT, spun at 15,000*g* for 20 min RT and the supernatant retained; samples could be frozen at −20 °C for further analysis.

2.3. Protein quantification

Protein quantification followed the method of Lowry et al. (1951) as modified by Price (1965). A stock of Lowry Reagent D was made up daily in a 48:1:1 ratio of Lowry Reagents A (2% (w/v) Na_2CO_3 (anhydrous) in 0.1 N NaOH); B (1% (w/v) NaK Tartrate tetrahydrate) and C (0.5% (w/v) $CuSO_4.5H_2O$ in H_2O), respectively. Reagents A, B, and C can be stored at RT. The Lowry assay also employs Folin-Ciocalteu phenol reagent (Sigma). A stock of a 1:1 ratio of 2 N Folin-Ciocalteu phenol regent: ultra-pure water was made daily. An appropriate volume (up to 50 μL) of the above protein extract was added to individual 1.5 mL microfuge tubes in triplicate, followed by 950 μL of Lowry Reagent D followed by immediate mixing (by inversion). Samples were then incubated for 10 min at RT. Next, 0.1 mL of the diluted Folin-Ciocalteu phenol reagent was added to each tube and vortexed immediately. After 30 min at RT, the absorbance of each sample was read at 600 nm (Nicolet Evolution 300 spectrophotometer, Thermo Electron Corporation, Madison, WI) using VisionPro™ software (Thermo Electron Corporation) (Findlay, 1990; Walker, 2002). Calibration curves were prepared for each assay with a bovine serum albumin (BSA) stock solution (200 mg/mL; Sigma P5369) and using a polynomial line of best fit generated in Microsoft Excel 2010.

2.4. Total organic nitrogen and carbon determination

Total organic carbon and nitrogen was also determined (ANCA-GSL 20-20 stable isotope analyzer, PDZ Europa, Sercon Ltd., Crewe, UK). Freeze-dried samples over the range 2–10 mg micro-algal DW biomass were combusted in pre-weighed aluminum capsules with helium as a carrier gas. The instrument was calibrated with L-isoleucine standards (Sigma) ranging from 5–200 μg N and 33–1320 μg C, delta calibrated N-7.89, C-12.18. Nitrogen to protein conversion ratios were determined as described by López et al. (2010).

2.5. Statistical analysis

Experimental error was determined for triplicate assays and expressed as standard deviation (SD). The significance of differences in the protein yield for micro-algal species subjected to varying TCA treatments and alkaline solution incubation was determined by *t*-test ($n = 3$). For comparison of total N-content and Lowry protein data, the Pearson correlation coefficient was determined using Minitab® Statistical Software version 15.0.

3. Results and discussion

In order to develop a rapid small-scale protein quantification method for lyophilized micro-algal material, a method based on hot-TCA extraction was chosen as a starting point (Price, 1965). The original method harvested fresh algal material by filtration (*E. gracilis*) and extracted without homogenization in 6% (w/v) hot-TCA (temperature unspecified) for 1 min. This was followed by centrifugation and incubation of the precipitated material with Lowry Reagent D (which contains 0.1 N NaOH, Section 2.3) at 55 °C for 3 min. Extracted protein could then be conveniently measured with a modified-Lowry assay, as described (Price, 1965).

For the method developed here, harvesting of cells by centrifugation of cells was carried out in place of filtration to avoid potential losses due to adsorption, highlighted by Rausch (1981). This also avoided the possible need to homogenize the filter and reduced handling time. Both the hot-TCA incubation and alkaline solution pellet-resuspension steps were considered to be important in determining yields in this procedure. It was also anticipated that extraction efficiency would vary according to species-specific factors. Therefore these two steps were evaluated in detail with seven micro-algal strains that covered a broad taxonomical range (Eustigmatophyceae, Chlorophyceae, Prasinophyceae, Trebouxiophyceae and Rhodophyceae) and were able to grow in variety of saline conditions (Table 1). The resultant optimized extraction procedure is shown as a diagram in Fig. 1.

3.1. Optimizing hot-TCA extraction

To define optimal conditions for extraction of proteins from lyophilized micro-algal material in hot-TCA, incubation temperature and TCA concentration were investigated in a matrix of four different conditions (Fig. 2). Hot-TCA extraction was tested at the original TCA concentration of Price (1965) at 6% (w/v) and fourfold higher at 24% (w/v). In the latter case, the TCA was diluted back to 6% (w/v) prior to centrifugation. It was found that lyophilized material could be readily resuspended in TCA by vortexing prior to the incubation steps in all seven strains. Incubation in TCA was tested at two different temperatures, 65 and 95 °C, for 15 min.

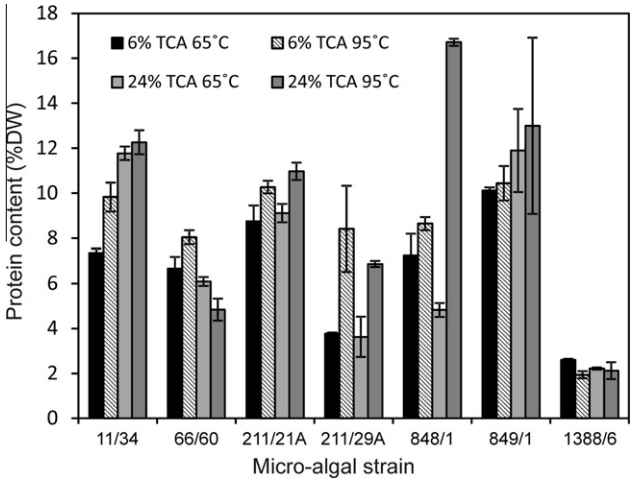


Fig. 2. The effect of temperature and TCA concentration on the efficiency of hot-TCA extraction in terms of protein yield (%DW). Seven different micro-algal species were subjected to four different combinations of hot-TCA conditions: 6% (w/v) TCA at 65 °C; 6% (w/v) 95 °C; 24% (w/v) 65 °C and 24% (w/v) 95 °C. Incubation was for 15 min followed by a 2 h solubilization of precipitated protein in Lowry Reagent D at 55 °C. Data are shown for the following micro-algal strains: *Dunaliella primolecta* (CCAP 11/34); *Tetraselmis* sp. (CCAP 66/60); *Chlorella ovalis* (CCAP 211/21A); *Chlorella spaerckii* (CCAP 211/29A); *Monodopsis subterranea* (CCAP 848/1); *Nannochloropsis oculata* (CCAP 849/1); *Rhodella violaceae* (CCAP 1388/6). Data are mean values of replicate assays, error bars indicate SD.

In Fig. 2, the effect of these treatments is shown in terms of protein yield improvement (%DW) for the seven micro-algal strains. Considerable increases in protein yield were observed for three of the seven strains either by raising TCA concentration to 24% (w/v) TCA and/or increasing incubation temperature to 95 °C (Fig. 2). In the case of the Eustigmatophycean micro-alga *Monodopsis subterraneae*, yields were doubled with the most stringent treatment (24% (w/v) TCA at 95 °C) relative to all the milder treatments ($P < 0.001$). Therefore both higher temperature and TCA concentration were needed to improve yields with this species. For the marine *Chlorella* species *Chlorella spaerckii*, increasing incubation temperature alone from 65 to 95 °C, at either TCA concentration, was sufficient to double yield ($P < 0.02$) relative to the mildest treatment (6% (w/v) TCA at 65 °C). With *Dunaliella primolecta*, a Chlorophyceaen micro-alga, incubation in higher concentrations of TCA (24%, v/v) at either temperature was sufficient to raise yields by 60–70% ($P < 0.001$) whereas a rise in temperature alone increased yield to a lesser degree (34%, $P < 0.01$). Therefore increasing TCA concentration and incubation temperature enhanced yields to differing degrees depending on the micro-algal species.

Taken together, the most stringent hot-TCA extraction condition (24% (w/v) TCA at 95 °C) produced substantial improvements in yield for three out of seven strains compared with milder treatments. This suggested that potentially, this treatment could be widely applicable across micro-algal taxa.

3.2. Evaluation of alkaline treatment

The next step in the Price (1965) procedure after hot-TCA extraction was recovery of precipitated protein and other insoluble material by centrifugation. In the original procedure, this was followed by re-solubilization of the centrifugal pellet in the alkaline Lowry Reagent D (0.1 N NaOH), for 3 min at 55 °C. It was noted that alkaline solutions are often used as protein extraction reagents in their own right (Section 1). Therefore in some circumstances, incomplete extraction with hot-TCA might be mitigated during the pellet-incubation period leading to yield maximization. Prolonged incubation times could reduce yield through degradation by hydrolysis, however (Rausch, 1981). In the above analysis of the hot-TCA conditions (Section 3.1), subsequent incubation of the TCA pellet in Lowry Reagent D was carried out at 55 °C for 2 h. To resolve these issues and optimize the procedure further, the incubation time period in Lowry Reagent D was investigated further (Figs. 3 and 4).

In the case of three strains (*D. primolecta*, *C. spaerckii* and *M. subterraneae*), it was previously established that the most stringent hot-TCA extraction condition (24% (w/v) TCA at 95 °C) provided significant improvements (Fig. 2). Therefore this treatment was followed by a range of incubation periods from 10 min to overnight (1320 min) in Lowry Reagent D (Fig. 3). Protein yields for *M. subterraneae* were found to be highly dependent on Lowry Reagent D incubation time, ranging from 6% to 19% DW. The yield curve, which tended towards an asymptote, suggested that most of the protein was resolubilized after the overnight incubation. This was consistent with further extraction of protein occurring with prolonged incubation. Nevertheless, sampling at 4 h was not significantly different ($P > 0.05$) from overnight incubation in terms of yield, suggesting 2–4 h as a possible compromise period for practical purposes. In contrast, length of incubation had no effect in the case of *C. spaerckii* and only a minor effect with *D. primolecta*,

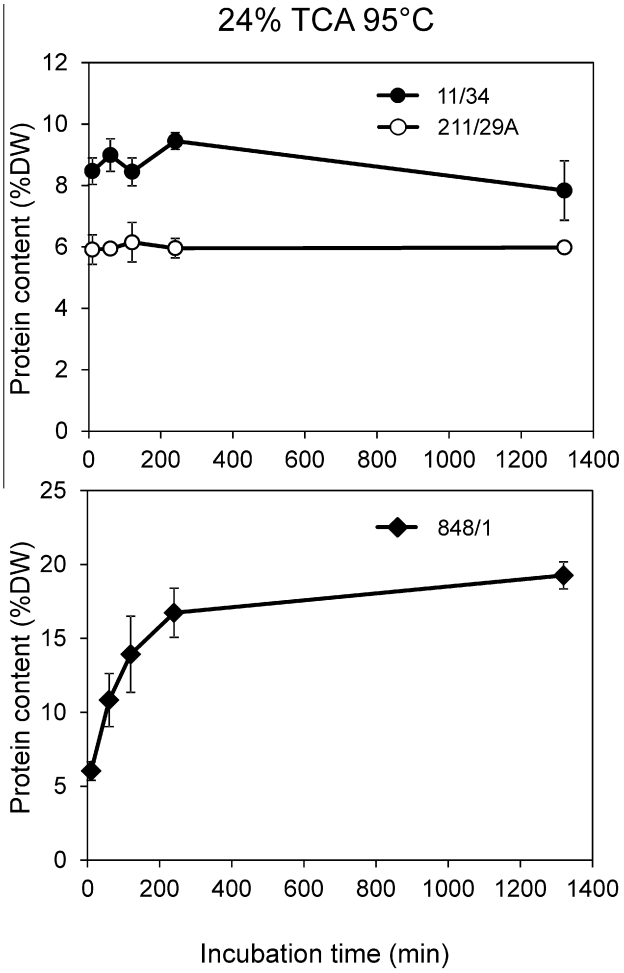


Fig. 3. The effect of incubation times on solubilization of TCA-pellets in Lowry Reagent D. Three micro-algal species were subjected to extraction in 24% (w/v) TCA at 95 °C for 15 min followed by increasing incubation periods in Reagent D at 55 °C. The following micro-algal strains were tested: *Dunaliella primolecta* (CCAP 11/34); *Chlorella spaerckii* (CCAP 211/29A) and *Monodopsis subterraneae* (CCAP 848/1). Data are mean values of replicate assays, error bars indicate SD.

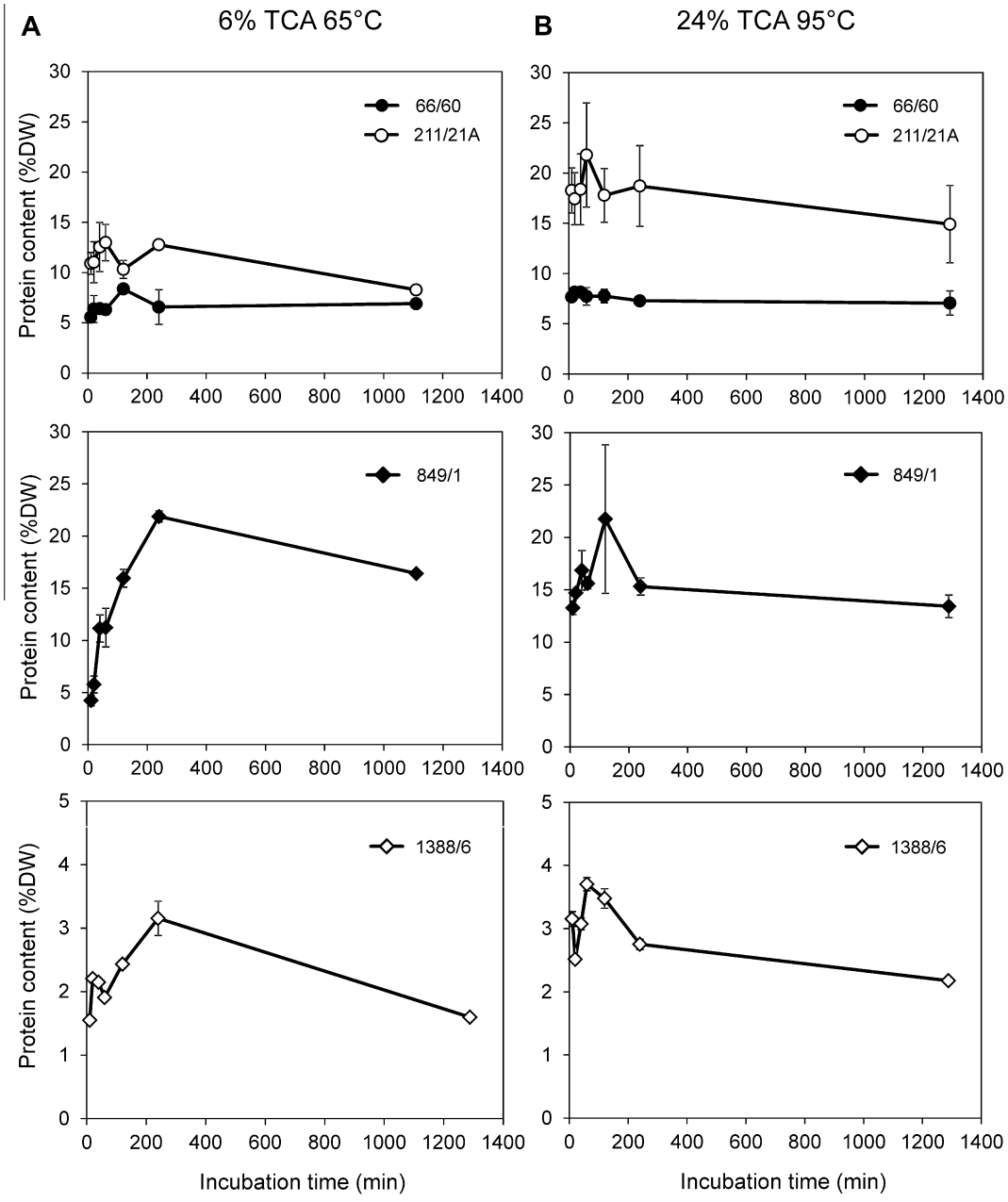


Fig. 4. The effect of incubation time on solubilization of TCA-pellets in Lowry Reagent D at two different prior hot-TCA treatments. Four micro-algal species were subjected to two different 15 min hot-TCA treatments: (A) 6% (w/v) TCA at 65 °C and (B) 24% (w/v) TCA at 95 °C. Following TCA precipitation, pellets were exposed to increasing incubation in Lowry Reagent D. The following micro-algal strains were tested: *Tetraselmis* sp. (CCAP 66/60); *Chlorella ovalis* (CCAP 211/21A); *Nannochloropsis oculata* (CCAP 849/1); *Rhodella violaceae* (CCAP 1388/6). Data are mean values of replicate assays, error bars indicate SD.

where the 4 h incubation period was optimal (10–20% higher yield: $P < 0.05$, except for 2 h).

A similar analysis of pellet-incubation period was carried out for the remaining four strains. Here yield was not found to be influenced strongly by the different hot-TCA conditions used in Fig. 2. Therefore these samples were extracted beforehand with both the lowest and highest stringency hot-TCA treatments (Fig. 4). These data show that for *Nannochloropsis oculata* and to a lesser extent *R. violaceae*, increasing incubation time in Lowry Reagent D from 10 min to 4 h, resulted in progressive yield increases, but only where the mildest hot-TCA treatment had been applied (Fig. 4A). This suggested that TCA protein extraction had been incomplete in the mild conditions, but could be completed with extended incubation in Lowry Reagent D. It followed that extraction under the most stringent TCA conditions was probably complete in these species, given that extended incubation in alkaline solution proved unnecessary (Fig. 4B).

In the case of *Chlorella ovalis* and *Tetraselmis* sp., extended incubations in Lowry Reagent D produced no improvements in yield (irrespective of hot-TCA pre-treatment stringency) (Fig. 4). In *Tetraselmis* sp., alterations in the hot-TCA extraction conditions or the pellet incubation time did not have a strong effect on yield (Fig. 4). In *C. ovalis*, yields were somewhat higher at 60–70% (10, 20, 60 and 120 min: $P < 0.05$), where the harshest TCA pre-treatment had been applied. Therefore, yield was dependent on the initial hot-TCA conditions and could not be improved in the subsequent alkaline solution step. This was in contrast to the Eustigmatophyceans *M. subterraneae* (Fig. 3) and *N. oculata*

Table 2
Nitrogen content determined by elemental analysis for seven species of marine micro-algae.

Micro-algal strain	CCAP No.	N-content		Protein content		N-conversion factor
		%DW	SD	%DW	SD	
Dunaliella primolecta	11/34	2.29	0.40	12.26	0.53	5.37
Tetraselmis sp.	66/60	1.50	0.16	4.83	0.49	3.23
Chlorella ovalis	211/21A	2.54	0.16	10.97	0.39	4.33
Chlorella spaerckii	211/29A	1.88	0.18	6.87	0.14	3.66
Monodopsis subterraneae	848/1	6.14	0.75	16.71	0.16	2.72
Nannochloropsis oculata	849/1	3.76	0.35	13.00	3.92	3.46
Rhodella violaceae	1388/6	0.71	0.08	2.12	0.38	3.00

N-content data: mean of replicate assays, standard deviation (SD) indicated. Protein content data from extraction in 24% (w/v) TCA at 95 °C, depicted in Fig. 2. N-conversion factor: Total N-content by elemental analysis to estimated protein content by Lowry assay.

(Fig. 4A) where incomplete extraction in hot-TCA could be overcome by extended incubation in Lowry Reagent D.

Overall, yield improvements were obtained by extending Lowry Reagent D incubation time but this was found to be species-dependent as noted for the hot-TCA conditions. Incubation of TCA-pellet in Lowry Reagent D beyond 4 h appeared to reduce yields with *D. primolecta* (Fig. 3), *R. violaceae*, *N. oculata* and *C. ovalis* (Fig. 4). This effect was not noted in *C. spaerckii*, *M. subterraneae* (Fig. 3) or *Tetraselmis* sp. (Fig. 4).

To summarize, substantial improvements to protein yield could be obtained by altering: (a) hot-TCA concentration and temperature and (b) incubation period in Lowry Reagent D. Species-specific differences were found in this respect, with the most recalcitrant strain being *M. subterraneae*. Nevertheless it was possible to arrive at an optimal procedure for screening of multiple strains from different micro-algal phyla (Fig. 1). Use of more stringent hot-TCA extractions (24% (w/v) TCA at 95 °C for 15 min) than those described in Price (1965), substantially increased yields in three out of seven strains relative to the mildest treatment. This included the marine *C. spaerckii* and the Eustigmatophycean strain *M. subterraneae* which belong to taxa renowned for small cell size and difficulties presented in extraction (Chiu et al., 2009; Doucha and Lívanský, 2008). Provided the most stringent hot-TCA extraction was used, extended TCA-pellet incubation in Lowry Reagent D beyond 10–20 min was not required except for one of the seven strains, *M. subterraneae*, where extended incubation of 2–4 h was needed to maximize yield. For four of the other strains under investigation, incubation beyond 4 h showed indications of yield reduction, possibly due to hydrolysis occurring in the absence of further protein extraction. Therefore, a compromise 3 h period was chosen for incubation in Lowry Reagent D.

3.3. Organic nitrogen content

To provide an independent evaluation of the protein extraction method, total organic nitrogen content was determined for the seven strains by elemental analysis (Table 2). In Table 2, organic nitrogen content is shown in comparison with the protein content values obtained using the most stringent hot-TCA extraction (24% (w/v) TCA at 95 °C) and 2 h pellet incubation in Lowry Reagent D (Fig. 2). A wide range of organic N-content (0.7–6.1% DW) was obtained, with *R. violaceae* being the lowest and *M. subterraneae* being the highest. The data shows a close linear correlation between N-content and protein content determined by the modified-Price procedure (Pearson correlation coefficient $r = 0.902$, $P = 0.005$).

The derived N-conversion factors range from 2.7 to 5.4 with a mean value of 3.68 (SD = 0.90). This value is similar to the total nitrogen to protein value obtained by similar means (i.e. using elemental analysis and Lowry) from a previous survey of other micro-algal species and cyanobacteria (4.44) (López et al., 2010). N-conversion values are generally found to be lower than the traditional factor of 6.25 due to the presence of non-protein N-containing compounds (Lourenço et al., 2004).

Comparison of the data obtained by the modified-Price procedure (Table 2) with published values for species from the same genera (Table 3) also supports the reliability of this method. Similar estimates were obtained to those in the literature, particularly where the Lowry assay had also been employed and where conditions were also at stationary phase (or under low N condi-

Table 3
Published measurements of micro-algal protein content determined for the same micro-algal species or genera described in the work.

Class	Genus	Species	Accession No.	Sampled culture conditions	Protein content		Method	Standard	References
					%DW	SD			
Chlorophyceae	*Dunaliella*	*tertioloecta*	CS-175	Late log-phase	20	2.3[a]	HPLC	–	Brown (1991)
			–	Stationary phase	11.4	0.99	Lowry	BSA	Barbarino and Lourenço (2005)
Prasinophyceae	*Tetraselmis*	*chui*	CS-26	Late log-phase	31	2.3[a]	HPLC	–	Brown (1991)
		suecica	CS-187	Late log-phase	31	2.3[a]	HPLC	–	Brown (1991)
			–	Log phase	34.25	–	Kjeldahl	–	Whyte (1987)
			–	Stationary phase	32.94	–	Kjeldahl	–	Whyte (1987)
			CS-187	Continuous (high N)	10–15	–	Lowry	BSA	D'Souza and Kelly (2000)
			CS-187	Continuous (low N)	5–7.5	–	Lowry	BSA	D'Souza and Kelly (2000)
Trebouxiophyceae	*Chlorella*	*vulgaris*	–	Late log-phase	24	–	Kjeldahl	–	Fowden (1954)
Eustigmatophyceae	*Nannochloropsis*	*oculata*	CS-179	Mid-late log-phase	20.2	–	HPLC	–	Volkman et al. (1993)
			CS-216	Mid-late log-phase	22.1	–	HPLC	–	Volkman et al. (1993)
			CS-179	Late log-phase	35	2.3[a]	HPLC	–	Brown (1991)
		salina	CS-190	Mid-late log-phase	17.8	–	HPLC	–	Volkman et al. (1993)
	Nannochloropsis sp.		–	Steady-state	28.8	0.63	Kjeldahl	–	Rebolloso-Fuentes et al. (2001)
			CS-246	Mid-late log-phase	21.2	–	HPLC	–	Volkman et al. (1993)

Standard deviation (SD) or [a]mean% coefficient of variation, indicated as reported. Culture conditions at sample harvest are indicated as described in references. Protein measurement method indicated, where HPLC refers to sum quantified amino acid residues. Conversion factor of 6.25 used with Kjeldahl.

tions: D'Souza and Kelly, 2000). For instance, *Tetraselmis* (5 cf. 5–8%DW) and *Dunaliella* (12 cf. 11%DW). The estimates were somewhat lower than the published values where Kjeldahl had been employed, or an estimation of protein content from total amino acid residues had been made (e.g. *Tetraselmis*: 5 cf. 31–33%DW; *Nannochloropsis*: 13 cf. 18–35%DW; *Chlorella*: 7–11 cf. 24%DW). In this case, the latter methods may have over-estimated protein content due to measurement of free amino acids, that are often present in substantial amounts in micro-algae (Brown, 1991) along with other non-protein N-compounds which are also detected by Kjeldahl (López et al., 2010; Lourenço et al., 2004).

The basis for using estimating protein content from N-content using a traditional conversion factor of 6.25 assumes that protein is the only N-source and that N comprises 16% (w/w) of protein (Barbarino and Lourenço, 2005). Given that amino acids and other N-containing compounds are present, it is possible to derive corrected conversion factors (as above) but these are strain-specific and influenced by growth conditions such as media N-content (D'Souza and Kelly, 2000; López et al., 2010; Lourenço et al., 2004). It is also possible to quantify some N-compounds such as nitrate and nucleic acids and subtract these from total N-measurements (Rebolloso-Fuentes et al., 2001). Corrective approaches such as these add to processing time, therefore dye-based methods such as Bradford and Lowry are probably more appropriate for high-throughput screening procedures (Bradford, 1976; Lowry et al., 1951).

Overall, the modified-Price method data correlated with N-content measurements and was similar to other published data using the Lowry method. This was supportive of complete protein extraction and measurement using the hot-TCA protocol developed here.

4. Conclusion

Measuring protein content in micro-algal material can be hampered by extraction difficulties, especially with lyophilized material. Often this is overcome by introducing labor-intensive homogenization steps. The aim was to develop a method with sequential hot-TCA and alkaline solution extractions that avoided this and was simpler to carry out. Seven strains were tested, including those recalcitrant to extraction. An optimized procedure was arrived at in terms of reagent concentration, temperature and incubation period. Despite species-specific differences in extraction efficiency, it proved possible to select a single set of conditions suitable for all the strains in the survey.

Acknowledgements

This work is supported by the BioMara project (www.biomara.ac.uk). The Biomara project is funded by the European Regional Development Fund through the INTERREG IVA Programme, Highlands and Islands Enterprise, Crown Estate, Northern Ireland Executive, Scottish Government and Irish Government.

References

Barbarino, E., Lourenço, S.O., 2005. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. J. Appl. Phycol. 17, 447–460.

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207–210.

Berges, J.A., Fisher, A.E., Harrison, P.J., 1993. A comparison of Lowry, Bradford and Smith protein assays using different protein standards and protein isolated from marine diatom *Thalassiosira pseudonana*. Mar. Biol. 115, 187–193.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brown, M.R., 1991. The amino acid and sugar composition of 16 species of microalgae used in mariculture. J. Exp. Mar. Biol. Ecol. 145, 79–99.

Carrapiso, A.I., García, C., 2000. Development in lipid analysis: some new extraction techniques and *in situ* transesterification. Lipids 35, 1167–1177.

Chiu, S.-Y., Kao, C.-Y., Tsai, M.-T., Ong, S.-C., Chen, C.-H., Lin, C.-S., 2009. Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. Bioresour. Technol. 100, 833–838.

Clayton, J.R., Dortch, Q., Thorensen, S.S., Ahmed, S.I., 1988. Evaluation of methods for the separation and analysis of proteins and free amino acids in phytoplankton samples. J. Plankton Res. 10, 341–358.

Conover, S.A.M., 1975. Partitioning of nitrogen and carbon in cultures of the marine diatom *Thalassiosira fluviatilis* supplied with nitrate, ammonium, or urea. Mar. Biol. 32, 231–246.

Day, J.G., Slocombe, S.P., Stanley, M.S., 2012. Overcoming biological constraints to enable the exploitation of microalgae for biofuels. Bioresour. Technol. 109, 245–251.

Doucha, J., Lívanský, K., 2008. Influence of processing parameters on disintegration of *Chlorella* cells in various types of homogenizers. Appl. Microbiol. Biotechnol. 81, 431–440.

D'Souza, F.M.L., Kelly, G.J., 2000. Effects of a diet of a nitrogen-limited alga (*Tetraselmis suecica*) on growth, survival and biochemical composition of tiger prawn (*Penaeus semisulcatus*) larvae. Aquaculture 181, 311–329.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Findlay, J.B.C., 1990. Purification of membrane proteins. In: Harris, E.L.V., Angal, S. (Eds.), Protein Purification Applications: A Practical Approach. Oxford University Press, Oxford, pp. 59–82.

Fowden, L., 1954. A comparison of the compositions of some algal proteins. Ann. Bot. 18, 257–266.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve) Gran. Can. J. Microbiol. 8, 229–239.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621–639.

Huntington, T., 2009. Use of wild fish and other aquatic organisms as feed in aquaculture – a review of practices and implications in Europe. In: Hasan, M.R., Halwart, M. (Eds.), Fish as Feed Inputs for Aquaculture: Practices, Sustainability and Implications. FAO Fisheries and Aquaculture Technical Paper No. 518, Rome, pp. 209–268.

Kjeldahl, J., 1883. Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. Z. Anal. Chem. 22, 366–382.

López, C.V.G., García, M. del C.C., Fernández, F.G.A., Bustos, C.S., Chisti, Y., Sevilla, J.M.F., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol. 101, 7587–7591.

Lourenço, S.O., Barbarino, E., Lavín, P.L., Marquez, U.M., Aidar, E., 2004. Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. Eur. J. Phycol. 39, 17–32.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Potvin, G., Zhang, Z., 2010. Strategies for high-level recombinant protein expression in transgenic microalgae: a review. Biotechnol. Adv. 28, 910–918.

Price, C.A., 1965. A membrane method for determination of total protein in dilute algal suspensions. Anal. Biochem. 12, 213–218.

Pruvost, J., Van Vooren, G., Le Gouic, B., Couzinet-Mossion, A., Legrand, J., 2011. Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel production. Bioresour. Technol. 102, 150–158.

Radakovits, R., Jinkerson, R.E., Fuerstenberg, S.I., Tae, H., Settlage, R.E., Boore, J.L., Posewitz, M.C., 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropis gaditana*. Nat. Commun. 3, 686. http://dx.doi.org/10.1038/ncomms1688.

Rausch, T., 1981. The estimation of micro-algal protein content and its meaning to the evaluation of algal biomass I. Comparison of methods for extracting protein. Hydrobiologia 78, 237–251.

Rebolloso-Fuentes, M.M., Navarro-Pérez, A., García-Camacho, F., Ramos-Miras, J.J., Guil-Guerrero, J.L., 2001. Biomass nutrient profiles of the microalga *Nannochloropsis*. J. Agric. Food Chem. 49, 2966–2972.

Rodolfi, L., Zittelli, G.C., Barsanti, L., Rosati, G., Tredici, M.R., 2003. Growth medium recycling in *Nannochloropsis* sp. mass cultivation. Biomol. Eng. 20, 243–248.

Stephens, E., Ross, I.L., Mussgnug, J.H., Wagner, L.D., Borowitzka, M.A., Posten, C., Kruse, O., Hankamer, B., 2010. Future prospects of microalgal biofuel production systems. Trends Plant Sci. 15, 554–564.

Volkman, J.K., Brown, M.R., Dunstan, G.A., Jeffrey, S.W., 1993. The biochemical composition of marine microalgae from the class Eustigmatophyceae. J. Phycol. 29, 69–78.

Walker, J.M., 2002. The Proteins Protocols Handbook, second ed. Humana Press Inc., Totowa, NJ.

Williams, P.J.leB., Laurens, L.M., 2010. Microalgae as biodiesel and biomass feedstocks: reviews and analysis of the biochemistry, energetics and economics. Energy Environ. Sci. 3, 554–590.

Whyte, J.N.C., 1987. Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. Aquaculture 60, 231–241.