Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications

Tibbetts, Sean M.; Whitney, Crystal G.; MacPherson, Margaret J.; Bhatti, Shabana; Banskota, Arjun H.; Stefanova, Roumiana; McGinn, Patrick J.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.algal.2014.11.011
Algal Research, 2014-12-19

Algal Research xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Algal Research

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

Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications

Sean M. Tibbetts *, Crystal G. Whitney, Margaret J. MacPherson, Shabana Bhatti, Arjun H. Banskota, Roumiana Stefanova, Patrick J. McGinn

National Research Council of Canada, Aquatic and Crop Resource Development, 1411 Oxford Street, Halifax, Nova Scotia B3H 3Z1, Canada

ARTICLE INFO

Article history:
Received 30 September 2014
Received in revised form 19 November 2014
Accepted 29 November 2014
Available online xxxx

Keywords:
Microalgae
Composition
Amino acids
Fatty acids
Minerals
Carotenoids
Animal feed

ABSTRACT

Biochemical composition of freshwater microalgae isolates from Alberta, Canada was determined. Growth rate (0.98 ± 0.07 d^{-1}), biomass production (0.35 ± 0.03 g DW L^{-1}) and daily productivity (0.14 ± 0.01 g DW L^{-1} d^{-1}) were the same for *Chlorella vulgaris* (AB02-C-U-BBM), *Nannochloris bacillaris* (AB03-C-F-PLM), *Tetracystis* sp. (AB04-C-F-PLM02) and *Micractinium reisseri* (AB05-C-U-BBM02). Whole algal biomass (WAB) contained low ash (~2 g 100 g DW^{-1}) and protein (13–15 g 100 g DW^{-1}), high esterifiable lipid (32–36 g 100 g DW^{-1}), carbohydrate (27–30 g 100 g DW^{-1}) and energy (26–28 MJ kg DW^{-1}). Oil fractionation was relatively ineffective for *C. vulgaris* and *M. reisseri* while oil was effectively extracted from *N. bacillaris* and *Tetracystis* sp. Accordingly, lipid-extracted biomass (LEB) from *N. bacillaris* and *Tetracystis* sp. contained higher protein (22 g 100 g DW^{-1}) and carbohydrate (43–44 g 100 g DW^{-1}) and lower residual esterifiable lipid (6–9 g 100 g DW^{-1}) than *C. vulgaris* and *M. reisseri* at 17–18 g 100 g DW^{-1} (protein), 34–36 g 100 g^{-1} DW (carbohydrate) and 28–32 g 100 g DW^{-1} (residual esterifiable lipid). Biomass had favorable essential amino acid (EAA) profiles with high EAA indices (0.9–1.1); rich in first-limiting EAA lysine (0.9–1.5 g 100 g DW^{-1}). Fatty acids (% of total) were predominantly monounsaturated fatty acids (MUFA; 40–53%), high in polyunsaturated fatty acids (PUFA; 27–40%) and low in saturated fatty acids (14–24%). *N. bacillaris* and *Tetracystis* sp. were particularly rich (9–12%) in α-linolenic acid (18:3n − 3), had attractive n − 3:n − 6 ratios (0.5–0.7:1), were rich in iron (800–1616 mg kg DW^{-1}), had attractively low Ca:P ratios (0.6–0.9:1) and were virtually absent of contaminating heavy metals.

1. Introduction

Microalgae are one of the most efficient organisms at transforming solar energy, carbon dioxide (CO_2) and inorganic elements into nutrient-rich biomass [1]. With a rapidly growing biofuel sector and expanding production of microalgae for other commercial purposes, it is expected that large quantities of algal products/co-products could become available in the near future [2]. Although algal oil for third-generation biodiesel production has been the subject of much research and a major driver for technological innovations in recent years, by all assessments it is not economically viable [3–5]. Utilization of the entire algal crop through a balanced biorefinery approach that effectively maintains the quality of various fractions has the potential to reduce the processing costs of each product and is likely the only feasible strategy to increase the viability of a microalgae industry [6–9]. At least in the near to mid-term, the livestock and aquaculture feed sectors appear to be among the most promising areas to focus for generating revenues [10,11]. The global animal feed market is currently valued at >$550 billion (b) USD annually (poultry, $69.9 b, swine, $89.3 b, cattle, $330.9 b, aquaculture, $60.5 b) and is continually seeking novel and sustainable sources of essential nutrients [12]. Depending on species/strain, environmental conditions and harvesting/processing methods, whole algal biomass and residual 'cake' after oil extraction may be highly attractive sources of essential dietary amino acids, fatty acids, sugars, vitamins, minerals, carotenoids and other health-promoting nutrients well suited as feeds or feed additives for terrestrial livestock and aquatic animals [1,13]. This potential for algal products/co-products for nutrition applications has long been recognized but commercial success has only been realized to a minor extent for a few species (e.g. *Spirulina*, *Chlorella*) occupying niche markets. The vast majority of this relatively small production (~15,000 t $year^{-1}$) is 'purpose-cultivated' as high value (>$10,000 t^{-1}) human nutritional supplements and pigments either in open cultivation ponds or open/closed hybrid systems [14]. Under the anticipated large-scale production of microalgae for biofuels and other commercial purposes, the residual biomass (e.g. defatted residues) will likely command a lower market value for animal feeds (<$1000 t^{-1}) than human nutritional supplements but, by sheer volume and potentially reduced processing costs, could dramatically increase the economic viability of industrial algaculture if marketed to the multi-billion dollar animal nutrition and aquaculture sectors [3,12].

* Corresponding author.
E-mail address: Sean.Tibbetts@nrc-cnrc.gc.ca (S.M. Tibbetts).

http://dx.doi.org/10.1016/j.algal.2014.11.011
2211-9264/

Dried whole algal biomass (WAB) can contain high levels of oil (generally up to 40%) that may be highly suitable as lipid supplements for animal feeds as a source of essential fatty acids (EFAs) and digestible energy (DE). Depending upon processing methods, the lipid-extracted biomass (LEB) remaining after oil extraction for other purposes (e.g., renewable energy) may contain significant levels of residual oil suitable for use in animal nutrition. Additionally, algal biomass from commercially-established species (e.g., *Chlorella*, *Spirulina*) generally contains all the essential amino acids (EAAs) in proportions more suitable to animal nutrition than many terrestrial plant-based crops in wide use such as corn, soybean, canola and wheat. As such, microalgae are highly attractive sources of essential nutrients and calories for animal feed applications [1,13]. Given good protein and lipid yields (both quantity and quality) of some algal species, good market potential and market price predictions, algal biomass could fall into the mid-value feed ingredient commodity sector (>$500 t^{-1}) presently dominated by terrestrial oilseed crops (e.g., soy, canola, corn) and could be sought after by the monogastric animal feed sector (e.g., poultry, swine, fish). Alternatively, if algal biomass is predominantly carbohydrate-rich, it will have a reduced market value similar to that of wheat and other cereal grain by-products (<$500 t^{-1}) and may be a better fit for the ruminant animal feed sector (e.g., cattle, sheep, deer). However, if it can be demonstrated that algal products have 'functional ingredient' properties (e.g., high response at low dose) their economic value may be considerably increased; provided that significant production tonnage can be economically achieved. In fact, it has been proposed that algal biomass could have certain market advantage over other terrestrial crops in terms of input costs (e.g. lower aerial foot-print, wastewater mitigation), carbon credits (e.g., industrial CO_2 conversion), nutritional value (e.g., more favorable EAA and EFA profiles) and raw ingredient sustainability [15]. As such, it is not surprising that algal products/co-products resulting from biofuel applications have been identified in Canada and elsewhere as a priority for further investigation as valuable commodities for sustainable development of terrestrial livestock and aquaculture feed inputs [16,17]. However, the nutritional content of algal biomass is poorly defined and for most species, including well-studied species like *Chlorella*, there is little consensus on their biochemical composition between and within species/strains and data on known and not-so-well known species isolated in Northern climates has not been reported, especially for LEB.

The present study is the first in a series of projects designed to evaluate the nutritional value of algal biomass produced from four freshwater species isolated in Northern Alberta, Canada for animal feed applications. Through an extensive microalgae isolation and screening program [18], these species have been identified as the most promising candidates for industrial carbon conversion in Northern climates, based on the following criteria: (1) freshwater and naturally-occurring in Alberta; (2) demonstrated high growth rate and esterifiable lipid productivity potential at flask level and (3) the inoculum was tolerant of simulated flue gas and grew well on treated wastewater at flask level. As such, they were mass cultivated in artificially illuminated 1000 L enclosed photobioreactors to produce sufficient biomass quantities for nutritional evaluation. The main objective of the present study was to generate novel primary biochemical composition data on whole algal biomass (WAB) and lipid-extracted biomass (LEB) including proximate, amino acid, fatty acid, carotenoid and elemental composition.

2. Materials and methods

2.1. Microalgae cultivation

This study investigated four microalgae species isolated from freshwater samples in Alberta, Canada [18] (Table 1). The isolates were identified by DNA sequence analysis of 18S, ITS1 and ITS2 regions (Eurofins Genomics, Huntsville, AL, USA) [19]. Species included *Chlorella vulgaris*, *Nannochloris bacillaris*, *Tetracystis* sp. and *Micractinium reisseri*. Unicellular cultures from our Canadian algae species collection were maintained immobilized on agar slants with standard Bold's basal medium [20] at 18–20 °C in continuous low light (50–75 μmol photons m^{-2} s^{-1} photosynthetically active radiation [PAR]). These algal library stocks are turned over onto fresh agar media on a monthly basis. Starter cultures were grown for one week on filter-sterilized (0.22 μm) f/2 media [20] in 250 mL flasks at 25 °C under 142 μmol photons m^{-2} s^{-1} PAR and agitated at 175 rpm on an orbital shaker. Aliquots of the flask cultures were diluted into 19 L carboys containing filtered (0.35 μm), pasteurized (85 °C for 6 h) and UV-sterilized freshwater. Filter-sterilized f/2 media was added to the carboys and cultivation was carried out at 22 °C and 100 μmol photons m^{-2} s^{-1} PAR for an additional week under aeration with filter-sterilized air. Final cultivation occurred in duplicate, proprietary enclosed 'Brite-Box' photobioreactors (PBRs) [21]. These are 1000 L internally illuminated PBRs enclosed by a fiberglass shell. Continuous light (250 μmol photons m^{-2} s^{-1} PAR) was provided by forty, F32T8/TL765 PLUS (32 W) fluorescent bulbs (Alto^{II}™ Technology, Philips Lighting, Markham, ON, Canada) arranged in eight horizontal rows of five. Approximately 980 L of membrane ultrafiltered (500 kDa nominal cut-off size) and UV-sterilized freshwater and filter-sterilized f/2 media were added to each PBR. Cultures were inoculated with microalgae from carboy cultures to an initial cell density of 45×10^4 cells mL^{-1} and maintained for 9–12 days at 22 °C with titanium heat-exchange cooling loops. Automated on-demand CO_2 injections maintained cultures at pH 7.0 and mixing was provided by aeration with a turbulent flow of sterilized air introduced into the cultures through perforations in two T-shaped air-lines situated medially at the bottom of the PBR. Cell densities were monitored daily, using a particle multi-sizer equipped with a 100 μm aperture tube (model MS3, Beckman-Coulter Inc., Miami, FL, USA). Growth rates (d^{-1}) were calculated as $\mu = ([\ln\{t_2\} - \ln\{t_1\}] / [t_2 - t_1])$ and biomass productivity as the product of the growth rate (μ) and the standing biomass density at time *t*.

Table 1
Microalgae species isolated from freshwater in Alberta, Canada[a].

Species	ID	Origin	Location
Chlorella vulgaris	AB02-C-U-BBM	Sylvan Lake	N52°18.867/W114°05.721
Nannochloris bacillaris	AB03-C-F-PLM	Athabasca River	N54°43.555/W113°17.109
Tetracystis sp.	AB04-C-F-PLM02	Pigeon Lake	N52°58.776/W114°02.151
Micractinium reisseri	AB05-C-U-BBM02	Gregoire Lake	N56°29.239/W111°10.833

[a] Stocks maintained immobilized on BBM agar media (18–20 °C; 50–75 μmol $m^{-2}s^{-1}$ light) in our Canadian algae species collection (Conviron environmental chamber, model PGR15, Winnipeg, MB, Canada).

2.2. Harvest and sample preparation

Cultures were harvested 6 days into stationary phase using a process centrifuge (model Z101, CEPA Carl Padberg Zentrifugenbau GmbH., Lahr, Germany) equipped with a 10 L collection chamber at 15,760 ×*g* and immediately frozen at −20 °C. Frozen paste (~25% solids) was lyophilized for 72 h at a low shelf temperature (<5 °C) in a large-capacity freeze-dryer (model 35EL, The Virtis Company, Gardiner, NY, USA) to a final moisture content of 3–4%. Freeze-dried whole algal biomass (WAB) was pulverized (to pass through a 0.5 mm screen) at 10,000 rpm using a laboratory ultra-centrifugal mill (Retsch model ZM200, Retsch GmbH., Haan, Germany) equipped with a pneumatic auto-feeder (Retsch model DR100) and stored at −80 °C. Lipid-extracted biomass (LEB) was produced by solvent extraction of WAB on a Soxtec™ automated system (model 2050, FOSS North America, Eden Prairie, MN, USA) using HPLC-grade $CHCl_3$:CH_3OH (2:1 v/v) at 150 °C for 82 min [22]. Residual solvents and moisture were eliminated

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

by drying LEB in an oven 50 °C for 24 h. Dried LEB from 60 pooled extractions per species were homogenized in a coffee grinder.

2.3. Analytical techniques

For each of the four microalgae species and the two fractions resulting from each (WAB and LEB), triplicate samples from duplicate cultures were analyzed for bulk density, proximate composition, caloric content, carotenoid composition, amino acid profile, fatty acid composition and elemental concentrations. Bulk density was measured according to CIPAC method MT186. Briefly, a known mass (13.0 g) of finely ground sample was placed into a glass measuring cylinder (50 mL) and then raised and allowed to fall vertically a distance of 2.5 cm onto a rubber pad ('tap'). After 50 taps within 1 min, the final volume was measured to calculate its bulk density (g cm^{-3}). Biochemical composition of algal biomass was conducted according to Laurens et al. [23] with minor alterations. Moisture and ash levels were determined gravimetrically after heating at 105 °C and after incineration at 550 °C, respectively until constant weight. Nitrogen (N) content was determined by elemental analysis (950 °C furnace) using a Leco N determinator (model FP-528, Leco Corporation, St. Joseph, MI, USA) with ultra-high purity oxygen as the combustion gas and ultra-high purity helium as the carrier gas. Amino acid concentrations were determined using the Waters Pico-Tag, reverse-phase high-performance liquid chromatography (PT-RP-HPLC) method [24,25]. Amino acid (AA) concentrations are expressed as mg AA g DW^{-1} and g AA 100 g $protein^{-1}$ and protein quality was evaluated on the basis of EAA indices [26]. Protein content was calculated using a nitrogen-to-protein conversion factor of N × 4.78 [27] and also by calculating species-specific nitrogen-to-protein conversion factors (N × *k*) for each species based on their individual amino acid contributions [28]. Fatty acids were extracted by methanolic HCl in-situ transesterification [29] and the corresponding fatty acid methyl esters (FAMEs) were separated and quantified by gas chromatography with flame ionization detection (Omegawax 250 column, Agilent 7890 GC-FID). Individual fatty acids, along with an internal standard (C19:0; methyl nonadecanoate, Fluka), were identified by comparing retention times to two standard mixtures (Supelco 37 and PUFA No. 3, Sigma-Aldrich). Total carbohydrate levels were determined by colorimetry using phenol and sulfuric acid [30]; following acid hydrolysis (2.5 M HCl at 80–90 °C for 3 h; [31]). Final results were determined against a dextrose standard curve (0–100 μg mL^{-1}; D-glucose, solid, >99% pure, Sigma-Aldrich, Cat. G5400). Starch content was determined by the α-amylase and amyloglucosidase method [32] using a Total Starch Assay Kit (K-TSTA, Megazyme International Ireland Ltd., Wicklow, Ireland) accepted by AOAC (Official Method 996.11) and AACC (Method 76.13). Fiber levels were estimated by difference (fiber = total carbohydrate − starch). Caloric content was measured as gross energy (MJ kg^{-1}) using an isoperibol oxygen bomb calorimeter (model 6200, Parr Instrument Company, Moline, IL, USA) equipped with a Parr 6510 water handling system for closed-loop operation. For determination of carotenoids, 10 mg of sample was extracted (×3) at room temperature with 5.0 mL of $CHCl_3$:MeOH (1:1 v/v) for 15 min followed by sonication (15 min) and the combined extracts were dried under N_2 gas. Extracts were then dissolved in 1.0 mL MeOH and stored at −20 °C prior to HPLC analysis. All carotenoid work was conducted under low light. Carotenoids analysis was performed using an Agilent 1200 series HPLC with a YMC Carotenoid column (5 μm, 2 × 250 mm, YMC Co., Ltd., Japan) eluted with 50 mM H_4OAc in MeOH-tertiary butyl methyl ether (TBME) linear gradients at 0.2 mL min^{-1} flow rate for 60 min. Standard curves of astaxanthin, α-carotene, β-carotene, canthaxanthin, fucoxanthin, lutein, lycopene and zeaxanthin at 450 nm were used for calculation of specific carotenoid levels. The concentrations of unknown carotenoids were calculated based on lutein as standard. Elemental composition was measured by inductively coupled argon plasma-optical emission spectrometry (ICP-OES) according to SW-846 Method 6010C [33]. Mercury was measured following reference method 7471B [33]. Briefly, 1 g of sample was digested for 75 min in concentrated HNO_3 at 95 °C after which, dissolution of organic matter was aided with the addition of H_2O_2 (30% v/v). Samples were then digested for an additional 1 h at 95 °C in concentrated HCl, made up to volume with reverse osmosis water and the concentration of mineral and trace elements determined using element-specific wavelengths on an IRIS Intrepid II spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). All analytical work was conducted in triplicate.

2.4. Statistical methods

Data is reported as mean ± standard deviation. Statistical analyses were performed using one-way analysis of variance, ANOVA (SigmaStat® v.3.5) with a 5% level of probability ($P < 0.05$) selected in advance to sufficiently demonstrate a statistically significant difference. Where significant differences were observed, treatment means were differentiated using pairwise comparisons using the Tukey test. Correlations between response variables were calculated by Pearson correlation analysis (*r*) using SigmaStat® v.3.5. Raw data was checked for normality using the Kolmogorov–Smirnov test (SigmaStat® v.3.5).

3. Results

3.1. Growth, productivity and bulk density

The growth rate, biomass productivity and bulk density of experimental cultures are shown in Table 2. Growth rates and productivities of *C. vulgaris*, *N. bacillaris*, *Tetracystis* sp. and *M. reisseri* were statistically the same at 0.9–1.0 d^{-1} ($P = 0.372$), 1.0–1.1 g paste L^{-1} ($P = 0.221$), 0.3–0.4 g DW L^{-1} ($P = 0.380$) and 0.1 g DW L^{-1} d^{-1} ($P = 0.528$). Significant differences ($P < 0.001$) were observed in bulk density between species; although the range is narrow (0.4–0.5 g cm^{-3}) and may not have much practical importance. *M. reisseri* had the highest bulk density (0.49) followed by *N. bacillaris* (0.45) and the lowest bulk densities were observed for *C. vulgaris* and *Tetracystis* sp. which had similar bulk densities (0.40–0.41; $P = 0.334$). Bulk density was directly correlated to the esterifiable lipid content of the biomass ($r = 0.76$; $P = 0.004$). In contrast, bulk density of the biomass was not significantly correlated to levels of protein ($r = 0.36$; $P = 0.250$) or carbohydrate ($r = 0.30$; $P = 0.353$).

3.2. Gross biochemical composition

Proximate composition and caloric content of whole algal biomass (WAB) and lipid-extracted biomass (LEB) of the four experimental species are shown in Table 3. Ash levels were consistently low for all biomass (<3 g 100 g DW^{-1}). Organic constituents (e.g. protein, lipid and carbohydrate) were the major components of WAB (>75% DW) with esterifiable lipid the most abundant component (32–36 g 100 g DW^{-1}), followed by carbohydrate (27–30 g 100 g DW^{-1}) and relatively low protein (13–15 g 100 g DW^{-1}). Although the ranges are relatively narrow, statistical differences were observed between species. When protein contents were calculated using a nitrogen-to-protein (N-to-P) conversion factor of N × 4.78, *M. reisseri* contained significantly higher protein (15 g 100 g DW^{-1}; $P < 0.001$) than *C. vulgaris* and *N. bacillaris* (14 g 100 g DW^{-1}; $P = 0.945$) and the lowest level was found in *Tetracystis* sp. (13 g 100 g DW^{-1}; $P = 0.001$). Calculating protein contents using 'species-specific' N-to-P conversion factors (N × *k*), however, showed all species having a similar amount of protein (15 g 100 g DW^{-1}; $P = 0.233$). *M. reisseri* contained significantly lower esterifiable lipid (32 g 100 g DW^{-1}; $P = 0.004$) than *C. vulgaris*, *N. bacillaris* and *Tetracystis* sp. which had statistically the same levels (35–36 g 100 g DW^{-1}; $P = 0.645$). Carbohydrate contents of *C. vulgaris* and *M. reisseri* were the same (30 g 100 g DW^{-1}; $P = 0.950$) and significantly higher ($P = 0.001$) than those of

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

Table 2
Growth, productivity and bulk density of microalgae species isolated from freshwater in Alberta, Canada[1].

Species	Growth rate (d^{-1})	Yield			
		Algal paste (g paste L^{-1})	Dry weight (g DW L^{-1})	Biomass productivity (g DW L^{-1} d^{-1})	Bulk density (g cm^{-3})
Chlorella vulgaris	0.9 ± 0.08^{ns}	1.0 ± 0.07^{ns}	0.3 ± 0.02^{ns}	0.1 ± 0.00^{ns}	0.41 ± 0.00^{a}
Micractinium reisseri	1.0 ± 0.07	1.0 ± 0.10	0.3 ± 0.01	0.1 ± 0.02	0.49 ± 0.00^{c}
Nannochloris bacillaris	1.0 ± 0.04	1.0 ± 0.01	0.4 ± 0.02	0.1 ± 0.02	0.45 ± 0.00^{b}
Tetracystis sp.	1.0 ± 0.06	1.1 ± 0.01	0.4 ± 0.01	0.1 ± 0.00	0.40 ± 0.00^{a}

[1] Values within the same column having different superscript letters are significantly different ($P < 0.05$).

N. bacillaris and *Tetracystis* sp. which were statistically the same (27–28 g 100 g DW^{-1}; $P = 0.787$). As a result of different lipid extraction efficiencies between *C. vulgaris* and *M. reisseri* and *N. bacillaris* and *Tetracystis* sp., different trends were observed for LEB. Oil fractionation was relatively ineffective for *C. vulgaris* and *M. reisseri* (28–32 g 100 g DW^{-1} residual esterifiable lipid) while oil was effectively extracted from *N. bacillaris* and *Tetracystis* sp. (6–9 g 100 g DW^{-1} residual esterifiable lipid). Accordingly, LEB from *N. bacillaris* and *Tetracystis* sp. contained significantly ($P < 0.001$) higher protein (22–24 g 100 g DW^{-1}) and carbohydrate (43–44 g 100 g DW^{-1}) than *C. vulgaris* and *M. reisseri* at 17–19 and 34–36 g 100 g DW^{-1}, respectively. Because oil removal effectively concentrates the non-lipid constituents of biomass, highly significant correlations were found between all macromolecular pools in LEB as follows: protein vs esterifiable lipid ($r = -0.99$; $P < 0.001$), protein vs carbohydrate ($r = 0.98$; $P < 0.001$) and esterifiable lipid vs carbohydrate ($r = -0.99$; $P < 0.001$). In addition to differences in total carbohydrate contents between species, differences in carbohydrate class were also observed. *M. reisseri* biomass contained significantly higher ($P < 0.001$) starch (19 g 100 g DW^{-1} [WAB]; 24 g 100 g DW^{-1} [LEB]) than *C. vulgaris* (15 g 100 g DW^{-1} [WAB]; 20 g 100 g DW^{-1} [LEB]). Starch contents of *N. bacillaris* and *Tetracystis* sp. biomass were low and statistically the same for WAB (1 g 100 g DW^{-1}; $P = 0.282$) and LEB (2–3 g 100 g DW^{-1}; $P = 0.380$). Since the major components of the macromolecular pool were in a relatively narrow range (protein, 13–15; esterifiable lipid, 32–36; carbohydrate, 27–30 g 100 g DW^{-1}) and comprised the majority of WAB (>75% DW), their caloric contents were also in a relatively narrow range (26–28 MJ kg DW^{-1}). Caloric content of LEB from *N. bacillaris* and *Tetracystis* sp. (21 MJ kg DW^{-1}; $P = 0.174$) was significantly lower ($P < 0.001$) than that of *C. vulgaris* and *M. reisseri* (24 MJ kg DW^{-1}; $P = 0.060$). Not surprisingly, caloric content of algal biomass was highly correlated with varying energy-yielding organic constituent levels including protein ($r = -0.97$; $P < 0.001$), esterifiable lipid ($r = 0.93$; $P < 0.001$) and carbohydrate ($r = -0.98$; $P < 0.001$).

Table 3
Proximate composition (g 100 g DW^{-1}) and caloric content (MJ kg DW^{-1}) of whole and lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.
Whole algal biomass				
Ash	2.4 ± 0.01^{a}	2.4 ± 0.01^{a}	1.9 ± 0.02^{b}	1.8 ± 0.01^{c}
Protein (N × 4.78)	13.8 ± 0.08^{b}	14.6 ± 0.08^{c}	13.8 ± 0.09^{b}	13.2 ± 0.19^{a}
Protein (N × *k*)	14.8 ± 0.08^{ns}	14.8 ± 0.08	14.9 ± 0.09	14.7 ± 0.21
Esterifiable lipid	34.8 ± 0.94^{b}	32.3 ± 1.31^{a}	35.4 ± 0.38^{b}	36.1 ± 0.56^{b}
Carbohydrate	29.8 ± 0.6^{b}	30.0 ± 0.7^{b}	27.2 ± 0.5^{a}	27.7 ± 0.7^{a}
Starch	15.4 ± 0.1^{b}	19.3 ± 0.1^{c}	1.3 ± 0.2^{a}	1.5 ± 0.1^{a}
Fiber	14.4 ± 0.7^{b}	10.7 ± 0.8^{a}	25.9 ± 0.5^{c}	26.2 ± 0.7^{c}
Gross energy	26.9 ± 0.04^{b}	26.3 ± 0.03^{a}	28.0 ± 0.05^{c}	28.3 ± 0.06^{d}
Lipid-extracted biomass				
Ash	2.7 ± 0.14^{ns}	2.6 ± 0.14	2.8 ± 0.09	2.7 ± 0.06
Protein (N × 4.78)	17.3 ± 0.32^{a}	17.7 ± 0.18^{a}	22.2 ± 0.01^{b}	21.9 ± 0.41^{b}
Protein (N × *k*)	18.8 ± 0.35^{a}	18.2 ± 0.19^{a}	23.3 ± 0.01^{b}	24.3 ± 0.45^{c}
Esterifiable lipid	31.8 ± 1.37^{d}	27.7 ± 1.13^{c}	6.1 ± 0.39^{a}	9.4 ± 1.76^{b}
Carbohydrate	33.6 ± 0.7^{a}	35.7 ± 0.9^{a}	43.9 ± 0.9^{b}	43.2 ± 0.9^{b}
Starch	20.1 ± 0.1^{b}	24.3 ± 0.6^{c}	2.5 ± 0.1^{a}	3.0 ± 0.0^{a}
Fiber	13.5 ± 0.8^{a}	11.4 ± 1.5^{a}	41.3 ± 1.0^{b}	40.2 ± 1.0^{b}
Gross energy	23.9 ± 0.37^{b}	24.5 ± 0.02^{b}	20.7 ± 0.22^{a}	21.2 ± 0.18^{a}

[1] Values within the same column having different superscript letters are significantly different ($P < 0.05$).

3.3. Fatty acid composition

Fatty acid composition of whole algal biomass (WAB) and lipid-extracted biomass (LEB) of the four experimental species are shown in Table 4. As a % of total fatty acid (FA), predominant FAs of all species were oleic acid (18:1n−9; 33–47%), linoleic acid (18:2n−6; 13–25%) and palmitic acid (16:0; 14–20%); representing ~77% of total FA (range, 73–82%). Fatty acids present at moderate levels were heptadecenoic acid (17:1; 3–5%), hexadecadienoic acid (16:2n−6; 4–8%) and α-linolenic acid (18:3n−3; 5–12%); representing ~18% of total FA (range, 14–20%). Fatty acids present at trace levels were stearic acid (18:0; 0–4%), hexadecenoic acid (16:1n−5; 0–1%), palmitoleic acid (16:1n−7; 0–2%), vaccenic acid (18:1n−7; 0–2%), hexadecatetraenoic acid (16:4n−3; 0–1%) and stearidonic acid (18:4n−3; 0–1%); representing ~4% of total FA (range, 1–6%). Algal FAs were highest in monounsaturates (MUFAs; 40–53%) followed by polyunsaturates (PUFAs; 27–40%) and lowest in saturates (SFAs; 14–24%) and highly significant differences ($P < 0.001$) were observed between species. *N. bacillaris* and *Tetracystis* sp. were higher in SFA (20–24%) than *M. reisseri* (17%) and *C. vulgaris* (14–15%). *C. vulgaris* was higher in MUFA (52–53%) than *N. bacillaris* and *Tetracystis* sp. (47–51%) and *M. reisseri* (40%). *M. reisseri* was higher in PUFA (37–40%) than *C. vulgaris* (32–33%) and *N. bacillaris* and *Tetracystis* sp. (27–30%). PUFA composition was significantly different ($P < 0.001$) between species with *M. reisseri* highest in n−6 PUFA (31–33%) and lowest in n−3 PUFA (6–7%). By contrast, *N. bacillaris* and *Tetracystis* sp. were lowest in n−6 PUFA (18%) and highest in n−3 PUFA (9–12%) and *C. vulgaris* was intermediate in terms of its n−6 PUFA (25%) and n−3 PUFA (6–7%). As a result, n−3:n−6 ratios followed accordingly: *N. bacillaris* and *Tetracystis* sp. (0.5–0.7:1) > *C. vulgaris* (0.3:1) > *M. reisseri* (0.2:1).

3.4. Carotenoid composition

Carotenoid composition of whole algal biomass (WAB) and lipid-extracted biomass (LEB) of the four experimental species are shown in Table 5. Total carotenoid levels of WAB were highest for *C. vulgaris* and *M. reisseri* (177–197 mg 100 g DW^{-1}) and lowest for *N. bacillaris* and *Tetracystis* sp. (130–145 mg 100 g DW^{-1}). The majority (67–79% of total carotenoid) was lutein (87–155 mg 100 g DW^{-1}), followed by moderate levels (12–25%) of zeaxanthin (21–36 mg 100 g DW^{-1}) and trace levels (3%) of fucoxanthin (6–7 mg 100 g DW^{-1}). With the exception of trace levels of lutein (7–21 mg 100 g DW^{-1}) in *C. vulgaris* and *M. reisseri*, LEB was free of carotenoids. Algal biomass produced from *C. vulgaris*, *M. reisseri*, *N. bacillaris* and *Tetracystis* sp. in this study, was

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

Table 4
Fatty acid composition (% of total FA) of whole and lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

Fatty acid	*Chlorella vulgaris*	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.
Whole algal biomass				
16:0	13.6 ± 0.10^{a}	16.5 ± 0.04^{b}	19.5 ± 0.01^{c}	20.0 ± 0.11^{d}
18:0	1.2 ± 0.02^{b}	0.4 ± 0.02^{a}	4.3 ± 0.01^{d}	4.2 ± 0.03^{c}
16:1n−5	nd[2]	1.3 ± 0.01	nd	nd
16:1n−7	1.9 ± 0.01^{d}	0.7 ± 0.01^{c}	0.4 ± 0.01^{a}	0.5 ± 0.01^{b}
17:1	4.3 ± 0.02^{b}	3.4 ± 0.07^{a}	4.6 ± 0.01^{c}	4.4 ± 0.07^{b}
18:1n−7	0.6 ± 0.01^{a}	1.4 ± 0.03^{d}	1.2 ± 0.01^{b}	1.3 ± 0.01^{c}
18:1n−9	45.2 ± 0.22^{c}	33.2 ± 0.49^{a}	41.0 ± 0.04^{b}	41.0 ± 0.24^{b}
16:2n−6	3.7 ± 0.02^{a}	8.2 ± 0.07^{c}	5.8 ± 0.01^{b}	5.8 ± 0.07^{b}
18:2n−6	21.5 ± 0.36^{b}	24.8 ± 0.28^{c}	12.7 ± 0.01^{a}	12.6 ± 0.11^{a}
16:4n−3	0.5 ± 0.01^{a}	0.6 ± 0.00^{b}	nd	nd
18:3n−3, ALA	7.5 ± 0.13^{b}	6.2 ± 0.08^{a}	9.5 ± 0.01^{d}	9.0 ± 0.12^{c}
18:4n−3	nd	nd	nd	nd
Other	0.2 ± 0.00^{a}	3.7 ± 0.24^{b}	0.9 ± 0.01^{a}	1.3 ± 0.71^{a}
Σ SFA	14.9 ± 0.12^{a}	16.8 ± 0.19^{b}	23.8 ± 0.01^{c}	24.2 ± 0.13^{d}
Σ MUFA	51.8 ± 0.21^{c}	40.0 ± 0.44^{a}	47.2 ± 0.03^{b}	47.2 ± 0.29^{b}
Σ PUFA	33.2 ± 0.12^{c}	39.6 ± 0.06^{d}	28.0 ± 0.02^{b}	27.3 ± 0.29^{a}
Σ n−6 PUFA	25.4 ± 0.27^{b}	33.0 ± 0.23^{c}	18.5 ± 0.01^{a}	18.3 ± 0.17^{a}
Σ n−3 PUFA	7.8 ± 0.19^{b}	6.6 ± 0.25^{a}	9.5 ± 0.01^{d}	9.0 ± 0.12^{c}
n−3:n−6 ratio	0.31 ± 0.01^{b}	0.20 ± 0.01^{a}	0.51 ± 0.00^{d}	0.49 ± 0.00^{c}
Lipid-extracted biomass				
16:0	14.1 ± 0.06^{a}	17.1 ± 0.01^{b}	19.5 ± 0.07^{c}	20.0 ± 0.58^{c}
18:0	nd	nd	nd	2.8 ± 0.40
16:1n−5	nd	nd	nd	nd
16:1n−7	2.0 ± 0.01^{c}	0.9 ± 0.04^{b}	0.6 ± 0.04^{a} nd	
17:1	3.9 ± 0.01^{d}	3.3 ± 0.01^{a}	3.6 ± 0.03^{c}	3.5 ± 0.11^{b}
18:1n−7	nd	2.5 ± 0.09	nd	nd
18:1n−9	47.1 ± 0.09^{b}	34.2 ± 1.47^{a}	46.9 ± 0.27^{b}	45.5 ± 0.86^{b}
16:2n−6	3.4 ± 0.02^{a}	7.1 ± 0.01^{c}	4.7 ± 0.03^{b}	4.7 ± 0.14^{b}
18:2n−6	21.1 ± 0.10^{b}	23.7 ± 0.02^{c}	13.3 ± 0.13^{a}	13.1 ± 0.74^{a}
16:4n−3	0.4 ± 0.01^{b}	0.4 ± 0.00^{a}	nd	nd
18:3n−3, ALA	6.3 ± 0.12^{b}	5.1 ± 0.10^{a}	11.6 ± 0.09^{c}	11.8 ± 0.37^{c}
18:4n−3	1.1 ± 0.13^{ns}	0.9 ± 0.10	nd	nd
Other	1.3 ± 0.96^{a}	5.1 ± 0.70^{b}	nd	nd
Σ SFA	14.1 ± 0.06^{a}	17.1 ± 0.01^{b}	19.5 ± 0.07^{c}	22.8 ± 0.79^{d}
Σ MUFA	52.9 ± 0.07^{d}	40.1 ± 0.07^{a}	50.9 ± 0.14^{c}	49.0 ± 0.97^{b}
Σ PUFA	32.3 ± 0.11^{b}	37.2 ± 0.03^{c}	29.6 ± 0.09^{a}	29.6 ± 0.76^{a}
Σ n−6 PUFA	24.5 ± 0.11^{b}	30.8 ± 0.02^{c}	18.0 ± 0.11^{a}	17.8 ± 0.84^{a}
Σ n−3 PUFA	7.8 ± 0.02^{b}	6.4 ± 0.01^{a}	11.6 ± 0.09^{c}	11.8 ± 0.37^{c}
n−3:n−6 ratio	0.32 ± 0.00^{b}	0.21 ± 0.00^{a}	0.65 ± 0.01^{c}	0.67 ± 0.05^{c}

[1] Values within the same column having different superscript letters are significantly different (P < 0.05).
[2] Not detected.

devoid of astaxanthin, canthaxanthin, α-carotene, β-carotene and lycopene.

3.5. Amino acid composition

Essential amino acid (EAA) and non-essential amino acid (NEAA) compositions (mg g DW^{-1}) of whole algal biomass (WAB) and lipid-extracted biomass (LEB) of the four experimental species are shown in Tables 6 and 7 and EAA compositions (g 100 g protein^{-1}) and EAA indices are shown in Table 8. Characteristic of microalgae, the NEAAs' aspartic acid and glutamic acid were predominant at 27–30 mg g DW^{-1} (WAB) and 34–51 mg g DW^{-1} (LEB); representing 3–5% of total DW. Algal biomass (WAB and LEB) was also rich in leucine (13–22 mg g DW^{-1}), alanine (11–18 mg g DW^{-1}), valine (9–16 mg g DW^{-1}), lysine (9–15 mg g DW^{-1}) and phenylalanine (8–16 mg g DW^{-1}). Algal biomass contained moderate levels of glycine (8–14 mg g DW^{-1}), proline (7–12 mg g DW^{-1}), isoleucine (6–12 mg g DW^{-1}), serine (6–12 mg g DW^{-1}), threonine (6–12 mg g DW^{-1}), tyrosine (5–9 mg g DW^{-1}) and tryptophan (3–8 mg g DW^{-1}). Amino acids detected at trace levels (<5 mg g DW^{-1}) included cysteine, histidine, taurine, methionine, α-amino-N-butyric acid, hydroxyproline and ornithine. Although

Table 5
Carotenoid composition (mg 100 g DW^{-1}) of whole and lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.
Whole algal biomass				
Total carotenoids	176.7 ± 12.6^{ab}	196.9 ± 9.9^{a}	145.3 ± 6.5^{bc}	130.2 ± 3.3^{c}
Astaxanthin	nd[2]	nd	nd	nd
Canthaxanthin	nd	nd	nd	nd
α-Carotene	nd	nd	nd	nd
β-Carotene	nd	nd	nd	nd
Fucoxanthin	6.2 ± 0.6^{ns}	6.9 ± 2.2	nd	nd
Lutein	132.7 ± 8.7^{a}	154.8 ± 10.2^{a}	96.8 ± 5.1^{b}	87.3 ± 3.0^{b}
Lycopene	nd	nd	nd	nd
Zeaxanthin	20.8 ± 2.0^{b}	23.9 ± 1.6^{b}	35.6 ± 1.4^{a}	32.3 ± 0.1^{a}
Other	17.0 ± 1.3^{a}	11.2 ± 0.2^{b}	12.8 ± 0.1^{b}	10.6 ± 0.1^{b}
Lipid-extracted biomass				
Total carotenoids	6.7 ± 1.7^{b}	20.9 ± 0.6^{a}	nd	nd
Astaxanthin	nd	nd	nd	nd
Canthaxanthin	nd	nd	nd	nd
α-Carotene	nd	nd	nd	nd
β-Carotene	nd	nd	nd	nd
Fucoxanthin	nd	nd	nd	nd
Lutein	6.7 ± 1.7^{b}	20.9 ± 0.6^{a}	nd	nd
Lycopene	nd	nd	nd	nd
Zeaxanthin	nd	nd	nd	nd
Other	nd	nd	nd	nd

[1] Values within the same column having different superscript letters are significantly different (P < 0.05).
[2] Not detected.

Table 6
Amino acid composition (mg g DW^{-1}) of whole algal biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.
Essential amino acids (EAAs)				
Arginine	9.8 ± 0.1^{b}	9.6 ± 0.1^{b}	9.2 ± 0.1^{a}	9.3 ± 0.0^{a}
Histidine	3.1 ± 0.2^{b}	2.9 ± 0.1^{b}	2.3 ± 0.0^{a}	2.3 ± 0.1^{a}
Isoleucine	6.5 ± 0.0^{a}	6.6 ± 0.1^{a}	7.8 ± 0.1^{c}	7.0 ± 0.1^{b}
Leucine	13.4 ± 0.1^{b}	13.9 ± 0.2^{c}	13.8 ± 0.3^{bc}	12.6 ± 0.1^{a}
Lysine	9.2 ± 0.3^{a}	12.7 ± 0.2^{b}	8.9 ± 0.2^{a}	9.1 ± 0.2^{a}
Methionine	3.5 ± 0.1^{c}	0.4 ± 0.0^{a}	2.8 ± 0.1^{b}	2.9 ± 0.0^{b}
Phenylalanine	8.2 ± 0.1^{a}	8.4 ± 0.1^{a}	8.8 ± 0.2^{b}	9.1 ± 0.2^{b}
Threonine	6.4 ± 0.2^{a}	6.5 ± 0.1^{a}	7.1 ± 0.0^{b}	7.4 ± 0.3^{b}
Tryptophan	2.7 ± 0.0^{a}	2.9 ± 0.1^{a}	6.6 ± 0.3^{b}	7.3 ± 0.1^{c}
Valine	9.0 ± 0.0^{b}	9.4 ± 0.1^{c}	9.7 ± 0.1^{d}	8.8 ± 0.1^{a}
Σ EAA	71.9 ± 0.9^{a}	73.4 ± 0.4^{b}	77.0 ± 0.3^{c}	75.9 ± 0.4^{c}
Non-essential amino acids (NEAAs)				
α-Amino-N-butyric acid	1.0 ± 0.0^{c}	0.9 ± 0.0^{c}	0.5 ± 0.0^{a}	0.6 ± 0.0^{b}
Aspartic acid	13.7 ± 0.3^{b}	12.6 ± 0.0^{a}	15.3 ± 0.2^{c}	12.9 ± 0.3^{a}
Alanine	11.2 ± 0.1^{b}	12.2 ± 0.2^{c}	10.7 ± 0.1^{a}	10.6 ± 0.0^{a}
Cysteine	2.4 ± 0.2^{b}	2.1 ± 0.1^{ab}	1.8 ± 0.1^{a}	2.4 ± 0.0^{b}
Glutamic acid	15.7 ± 0.5^{c}	14.6 ± 0.1^{ab}	15.1 ± 0.2^{bc}	14.4 ± 0.2^{a}
Glycine	8.6 ± 0.1^{bc}	8.8 ± 0.2^{c}	7.8 ± 0.1^{a}	8.5 ± 0.1^{b}
Hydroxyproline	0.7 ± 0.0^{b}	0.9 ± 0.0^{c}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}
Ornithine	nd	nd	0.1 ± 0.0^{ns}	0.1 ± 0.1
Proline	7.8 ± 0.1^{c}	8.2 ± 0.1^{d}	6.8 ± 0.1^{a}	7.3 ± 0.0^{b}
Serine	7.5 ± 0.1^{d}	7.3 ± 0.1^{c}	6.5 ± 0.1^{a}	7.0 ± 0.1^{b}
Taurine	1.1 ± 0.1^{a}	1.4 ± 0.1^{b}	1.1 ± 0.0^{a}	1.0 ± 0.0^{a}
Tyrosine	6.4 ± 0.2^{b}	6.1 ± 0.2^{b}	5.7 ± 0.0^{a}	5.5 ± 0.0^{a}
Σ NEAA	76.4 ± 1.2^{b}	75.1 ± 0.7^{b}	71.7 ± 0.7^{a}	70.7 ± 0.5^{a}
ΣAA	148.2 ± 2.0^{ns}	148.5 ± 1.2	148.7 ± 0.7	146.6 ± 0.9
EAA:NEAA ratio	0.94 ± 0.01^{a}	0.98 ± 0.00^{b}	1.07 ± 0.01^{c}	1.07 ± 0.00^{c}
k[2]	5.14	4.86	5.14	5.29

[1] Values within the same column having different superscript letters are significantly different (P < 0.05).
[2] Species-specific nitrogen-to-protein conversion factors calculated according to Mossé [28].

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

Table 7
Amino acid composition (mg g DW^{-1}) of lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.
Essential amino acids (EAAs)				
Arginine	12.3 ± 0.3^{b}	11.2 ± 0.4^{a}	13.6 ± 0.5^{c}	15.7 ± 0.3^{d}
Histidine	3.9 ± 0.0^{b}	3.4 ± 0.1^{a}	4.1 ± 0.2^{b}	3.8 ± 0.2^{ab}
Isoleucine	8.1 ± 0.1^{a}	8.2 ± 0.0^{a}	12.4 ± 0.6^{b}	11.7 ± 0.3^{b}
Leucine	17.3 ± 0.3^{a}	17.6 ± 0.1^{a}	22.1 ± 0.1^{b}	21.7 ± 0.5^{b}
Lysine	11.9 ± 0.2^{a}	14.3 ± 0.0^{b}	14.6 ± 0.4^{bc}	15.1 ± 0.0^{c}
Methionine	4.5 ± 0.1^{b}	0.5 ± 0.0^{a}	0.6 ± 0.0^{a}	4.7 ± 0.2^{b}
Phenylalanine	10.5 ± 0.3^{a}	10.5 ± 0.1^{a}	15.5 ± 0.1^{b}	16.0 ± 0.1^{c}
Threonine	8.2 ± 0.1^{a}	8.1 ± 0.1^{a}	11.8 ± 0.6^{b}	12.0 ± 0.2^{b}
Tryptophan	3.0 ± 0.1^{a}	3.2 ± 0.2^{a}	2.5 ± 0.5^{a}	8.1 ± 0.6^{b}
Valine	11.7 ± 0.1^{a}	12.0 ± 0.0^{a}	15.7 ± 0.3^{c}	15.1 ± 0.3^{b}
Σ EAA	91.4 ± 1.2^{a}	89.0 ± 0.8^{a}	113.1 ± 0.8^{b}	123.9 ± 1.2^{c}
Non-essential amino acids (NEAAs)				
α-Amino-N-butyric acid	0.9 ± 0.0^{d}	0.6 ± 0.0^{c}	0.3 ± 0.0^{a}	0.4 ± 0.0^{b}
Aspartic acid	17.4 ± 0.6^{a}	15.8 ± 0.1^{a}	25.1 ± 0.7^{c}	21.3 ± 1.4^{b}
Alanine	14.5 ± 0.2^{a}	15.3 ± 0.1^{a}	17.7 ± 0.8^{b}	17.9 ± 0.6^{b}
Cysteine	3.3 ± 0.1^{b}	2.5 ± 0.0^{a}	3.4 ± 0.1^{b}	4.6 ± 0.1^{c}
Glutamic acid	20.2 ± 0.8^{a}	18.5 ± 0.3^{a}	25.9 ± 1.1^{b}	24.9 ± 0.4^{b}
Glycine	11.2 ± 0.2^{a}	11.2 ± 0.1^{a}	13.9 ± 0.1^{b}	14.1 ± 0.2^{b}
Hydroxyproline	0.9 ± 0.0^{b}	1.1 ± 0.0^{c}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}
Ornithine	nd	nd	0.2 ± 0.0^{ns}	0.2 ± 0.0
Proline	10.0 ± 0.1^{a}	10.3 ± 0.2^{a}	11.9 ± 0.1^{b}	12.2 ± 0.3^{b}
Serine	9.7 ± 0.2^{a}	9.2 ± 0.1^{a}	11.5 ± 0.1^{b}	11.7 ± 0.4^{b}
Taurine	1.0 ± 0.0^{a}	1.1 ± 0.0^{b}	0.9 ± 0.0^{a}	2.1 ± 0.0^{c}
Tyrosine	7.9 ± 0.4^{ab}	7.3 ± 0.4^{a}	8.7 ± 0.3^{bc}	9.3 ± 0.1^{c}
Σ NEAA	97.1 ± 2.5^{a}	93.0 ± 0.7^{a}	120.0 ± 2.8^{b}	119.1 ± 3.3^{b}
ΣAA	188.5 ± 3.5^{a}	182.1 ± 1.2^{a}	233.1 ± 3.6^{b}	243.0 ± 4.4^{c}
EAA:NEAA ratio	0.94 ± 0.01^{a}	0.96 ± 0.01^{a}	0.94 ± 0.02^{a}	1.04 ± 0.02^{b}
k[2]	5.21	4.91	5.01	5.30

[1] Values within the same column having different superscript letters are significantly different (P < 0.05).
[2] Species-specific nitrogen-to-protein conversion factors calculated according to Mossé [28].

total AA contents of WAB from the 4 species were statistically the same (147–149 mg g DW^{-1}; P = 0.243), *N. bacillaris* and *Tetracystis* sp. had higher total EAA content (76–77 mg g DW^{-1}) than *C. vulgaris* and *M. reisseri* (72–73 mg g DW^{-1}) and lower total NEAA content (71–72 vs 75–76 mg g DW^{-1}, respectively). As a result, EAA/NEAA ratios for WAB followed accordingly: *N. bacillaris* and *Tetracystis* sp. (1.07) > *M. reisseri* (0.98) > *C. vulgaris* (0.94). With regard to LEB, *Tetracystis* sp. contained the highest total EAA (124 mg g DW^{-1}); followed by *N. bacillaris* (113 mg g DW^{-1}) and the lowest total EAA was observed for *C. vulgaris* and *M. reisseri* (89–91 mg g DW^{-1}). A similar trend was observed for total NEAA where *N. bacillaris* and *Tetracystis* sp. levels (119–120 mg g DW^{-1}) were significantly higher than those of *C. vulgaris* and *M. reisseri* (93–97 mg g DW^{-1}). As a result, EAA/NEAA ratios for LEB followed accordingly: *Tetracystis* sp. (1.04) > *M. reisseri* (0.96) = *N. bacillaris* (0.94) = *C. vulgaris* (0.94). Table 8 provides EAA composition data normalized to total protein levels (g 100 g $protein^{-1}$). This representation allows for more direct EAA profile comparisons (regardless of varying total protein levels) between species and with other common feed/food ingredients and is also required for calculation of EAA indices which are useful as an approximation of protein quality. All biomass (WAB and LEB) had highly favorable EAA profiles; rich in leucine (9–10 g 100 g $protein^{-1}$), lysine (6–9 g 100 g $protein^{-1}$) and tryptophan (2–5 g 100 g $protein^{-1}$). EAA indices were high for all biomass (0.9–1.1) but particularly *Tetracystis* sp. (1.0–1.1) which surpassed reference proteins (soy protein, 0.9; egg albumin protein, 1.0). EAA indices of WAB from *Tetracystis* sp. (1.10) was significantly higher (P = 0.008) than *N. bacillaris* (1.07). WAB of both *Tetracystis* sp. and *N. bacillaris* had significantly higher EAA indices (P < 0.001) than *C. vulgaris* and *M. reisseri* which were statistically the same (0.92–0.93; P = 0.384). Similarly, EAA indices of LEB from *Tetracystis* sp. (1.02) was significantly higher (P < 0.001) than those of all other species, with *C. vulgaris* and *M. reisseri* statistically the same (0.90–0.92; P = 165) and higher than *N. bacillaris* (0.86; P = 0.006).

3.6. Elemental composition

Elemental composition of whole algal biomass (WAB) and lipid-extracted biomass (LEB) of the four experimental species are shown in Tables 9 and 10. Minerals assayed included calcium (Ca), magnesium (Mg), phosphorous (P), potassium (K) and sodium (Na). Trace elements

Table 8
Essential amino acid (EAA) composition (g EAA 100 g $protein^{-1}$) and EAA indices of whole and lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.	Ref proteins [57]	
					Egg albumin	Soybean
Whole algal biomass						
Arginine	6.6 ± 0.1^{c}	6.5 ± 0.1^{bc}	6.2 ± 0.1^{a}	6.4 ± 0.0^{ab}	6.2	7.4
Histidine	2.1 ± 0.1^{b}	2.0 ± 0.1^{b}	1.5 ± 0.0^{a}	1.6 ± 0.0^{a}	2.4	2.6
Isoleucine	4.4 ± 0.0^{a}	4.4 ± 0.1^{a}	5.2 ± 0.1^{c}	4.8 ± 0.1^{b}	6.6	5.3
Leucine	9.0 ± 0.1^{b}	9.4 ± 0.1^{c}	9.3 ± 0.2^{bc}	8.6 ± 0.1^{a}	8.8	7.7
Lysine	6.2 ± 0.2^{a}	8.6 ± 0.1^{b}	6.0 ± 0.1^{a}	6.2 ± 0.1^{a}	5.3	6.4
Methionine	2.4 ± 0.1^{c}	0.3 ± 0.0^{a}	1.9 ± 0.1^{b}	2.0 ± 0.0^{b}	3.2	1.3
Phenylalanine	5.5 ± 0.0^{a}	5.6 ± 0.0^{ab}	5.9 ± 0.2^{bc}	6.2 ± 0.1^{c}	5.8	5.0
Threonine	4.3 ± 0.1^{a}	4.4 ± 0.0^{a}	4.8 ± 0.0^{b}	5.0 ± 0.2^{b}	5.0	4.0
Tryptophan	1.8 ± 0.0^{a}	1.9 ± 0.1^{a}	4.5 ± 0.2^{b}	5.0 ± 0.0^{c}	1.7	1.4
Valine	6.1 ± 0.1^{a}	6.3 ± 0.0^{b}	6.5 ± 0.0^{c}	6.0 ± 0.0^{a}	7.2	5.3
EAA indices	0.93 ± 0.01^{a}	0.92 ± 0.01^{a}	1.07 ± 0.01^{b}	1.10 ± 0.00^{c}	1.0	0.9
Lipid-extracted biomass						
Arginine	6.5 ± 0.2^{b}	6.2 ± 0.2^{ab}	5.9 ± 0.3^{a}	6.4 ± 0.1^{ab}		
Histidine	2.1 ± 0.0^{c}	1.9 ± 0.1^{b}	1.8 ± 0.1^{b}	1.6 ± 0.1^{a}		
Isoleucine	4.3 ± 0.0^{a}	4.5 ± 0.0^{a}	5.3 ± 0.2^{c}	4.8 ± 0.0^{b}		
Leucine	9.2 ± 0.0^{b}	9.6 ± 0.1^{d}	9.5 ± 0.1^{c}	8.9 ± 0.0^{a}		
Lysine	6.3 ± 0.1^{a}	7.9 ± 0.1^{b}	6.3 ± 0.2^{a}	6.2 ± 0.1^{a}		
Methionine	2.4 ± 0.0^{c}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	1.9 ± 0.1^{b}		
Phenylalanine	5.6 ± 0.0^{a}	5.7 ± 0.0^{a}	6.6 ± 0.1^{b}	6.6 ± 0.1^{b}		
Threonine	4.4 ± 0.0^{a}	4.5 ± 0.0^{a}	5.1 ± 0.2^{b}	5.0 ± 0.2^{b}		
Tryptophan	1.6 ± 0.1^{b}	1.8 ± 0.1^{b}	1.1 ± 0.2^{a}	3.3 ± 0.3^{c}		
Valine	6.2 ± 0.0^{a}	6.6 ± 0.0^{b}	6.8 ± 0.1^{b}	6.2 ± 0.1^{a}		
EAA indices	0.92 ± 0.01^{b}	0.90 ± 0.01^{b}	0.86 ± 0.01^{a}	1.02 ± 0.01^{c}		

[1] Values within the same column having different superscript letters are significantly different (P < 0.05).

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

assayed included copper (Cu), iron (Fe), manganese (Mn), selenium (Se) and zinc (Zn). Heavy metals assayed included arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg). For both WAB and LEB, *C. vulgaris* was generally the richest source of Ca (0.36–0.49 g 100 g DW^{-1}) and Mg (0.11 g 100 g DW^{-1}) and *N. bacillaris* the lowest in these minerals (0.16–0.26 and 0.08 g 100 g DW^{-1}, respectively). *Tetracystis* sp. and *M. reisseri* had intermediate levels of 0.20–0.42 and 0.05–0.09 g 100 g DW^{-1}, respectively. *M. reisseri* was generally the richest source of P (0.31–0.35 g 100 g DW^{-1}) and K (0.51–0.53 g 100 g DW^{-1}) with the other strains at similar but generally lower levels (0.27–0.40 and 0.40–0.55 g 100 g DW^{-1}, respectively). Ca:P ratios were significantly different ($P < 0.001$) between species with the same trend for both WAB and LEB as follows: *C. vulgaris* WAB (1.3–1.5) > *M. reisseri* WAB (1.0–1.2) > *Tetracystis* sp. WAB (0.7–0.9) > *N. bacillaris* WAB (0.6). The same trend was found for LEB as follows: *C. vulgaris* LEB (1.3–1.5) > *M. reisseri* LEB (1.0–1.2) > *Tetracystis* sp. LEB (0.7–0.9) > *N. bacillaris* LEB (0.6). With regard to WAB, *C. vulgaris* and *N. bacillaris* had the same levels of Cu (22 mg kg DW^{-1} $P = 1.000$); which was significantly higher ($P = 0.002$) than *M. reisseri* and *Tetracystis* sp. which had the same levels (13–14 mg kg DW^{-1}; $P = 0.818$). A similar trend was found for LEB as follows: *C. vulgaris* and *N. bacillaris* (32–36 mg kg DW^{-1}) ≥ *Tetracystis* sp. (29 mg kg DW^{-1}) > *M. reisseri* (22 mg kg DW^{-1}). Significant differences ($P < 0.001$) were found in the Fe content between species and the trend was the same for both WAB and LEB as follows: *Tetracystis* sp. (884 and 1616 mg kg DW^{-1}, respectively) > *N. bacillaris* (800 and 1312 mg kg DW^{-1}, respectively) > *M. reisseri* (257 and 346 mg kg DW^{-1}, respectively) > *C. vulgaris* (198 and 264 mg kg DW^{-1}, respectively). Significant differences ($P < 0.001$) were found in the Mn content between species and the trend was the same for both WAB and LEB as follows: *C. vulgaris* (35 and 44 mg kg DW^{-1}, respectively) > *M. reisseri* (32 and 40 mg kg DW^{-1}, respectively) > *Tetracystis* sp. (19 and 33 mg kg DW^{-1}, respectively) > *N. bacillaris* (17 and 27 mg kg DW^{-1}, respectively). *C. vulgaris* WAB and LEB had the highest Zn levels (25 and 35 mg kg DW^{-1}, respectively) which were significantly higher ($P < 0.001$) than the other species which had similar levels (11 and 18–23 mg kg DW^{-1}, respectively). Biomass of all algal species was low or devoid of Se (<0.6 mg kg DW^{-1}). Algal biomass was virtually absent of contaminating heavy metals such as As (<0.5 ppm), Cd (not detected) and Pb (<0.4 ppm). Although Hg was detected in every sample, levels were extremely low (0.003–0.008 ppm).

4. Discussion

Despite decades of study on the biotechnological applications of certain microalgae species, strategic research on mass algaculture for large industrial applications like renewable energy and nutrition for most species remains in its formative years and many questions and bottlenecks remain [34]. Vast diversity in species/strains, isolate geographical environment, culture conditions, harvesting/processing techniques and poorly defined biomass characterization methods have resulted in data that is difficult to reproduce and confidently apply to industrial applications [1,23,35–37]. The present study generated new data on the biochemical composition of four novel freshwater microalgae isolates identified as promising candidates for industrial CO_2 conversion in Northern climates. Under identical culture conditions in 1000 L, artificially illuminated, enclosed photobioreactors, the species were virtually indistinguishable in terms of growth rates (0.9–1.0 d^{-1}) and biomass productivities (1.0–1.1 g paste L^{-1}, 0.3–0.4 g DW L^{-1}; 0.1 g DW L^{-1} d^{-1}) and there appeared to be little differences with regard to their appearance, texture and smell; all of which have important implications for animal feeding [38–41]. Recent studies have shown that while the nutritional value of certain algal biomass for animal feeds may be as high (or even exceed) conventional plant sources (e.g., soy and corn), recommended dietary inclusion levels for certain farmed animals (e.g., swine and poultry) have been minimized (<10% of the diet) due to low feed intake as a result of unattractive smell and off-flavors [17,42].

A particular aspect of algal biomass evaluation for animal feeding applications that has been largely overlooked is 'bulk density'. Bulk density (BD) is a measure of the mass-to-volume ratio of a commodity ingredient or complete feed and is reported in either imperial (e.g., lbs ft^{-3} and lbs $bushel^{-1}$) or metric units (e.g., kg hL^{-1} and g cm^{-3}). Although BD has been used as a probable measure of metabolizable energy content (e.g., anabolic calories) of feed ingredients in the past [43], it has fallen out of favor for nutritional evaluation due to the variability between farmed animal species in their ability to digest and metabolize dietary energy-yielding nutrients. However, BD values still serve an important logistical role today because highly complex feed processing and distribution systems are generally designed around conventional ingredients with well-established and predictable physical characteristics; in particular BD. A large proportion of commercially-available feed ingredients used in the agriculture/aquaculture industries have a BD of 0.4–0.6 g cm^{-3} [44–48] and the algal biomass produced for this study falls

Table 9
Elemental composition of whole algal biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.	DL[2] (μg g^{-1})
Minerals (g 100 g DW^{-1})					
Calcium	0.36 ± 0.03^{c}	0.30 ± 0.01^{b}	0.16 ± 0.01^{a}	0.20 ± 0.02^{a}	2
Magnesium	0.11 ± 0.00^{d}	0.09 ± 0.00^{c}	0.08 ± 0.00^{b}	0.05 ± 0.00^{a}	1
Phosphorous	0.28 ± 0.00^{a}	0.31 ± 0.00^{b}	0.28 ± 0.01^{a}	0.27 ± 0.01^{a}	0.5
Potassium	0.40 ± 0.01^{a}	0.51 ± 0.01^{c}	0.44 ± 0.01^{b}	0.40 ± 0.01^{a}	5
Sodium	0.04 ± 0.00^{b}	0.05 ± 0.00^{c}	0.04 ± 0.00^{b}	0.03 ± 0.00^{a}	1
Ca:P ratio	1.28 ± 0.08^{d}	0.97 ± 0.04^{c}	0.56 ± 0.03^{a}	0.75 ± 0.04^{b}	–
Trace elements (mg kg DW^{-1})					
Copper	21.9 ± 1.8^{b}	14.5 ± 0.2^{a}	22.0 ± 4.3^{b}	12.8 ± 0.3^{a}	0.05
Iron	198.0 ± 6.0^{a}	256.8 ± 4.1^{b}	800.4 ± 19.7^{c}	883.6 ± 30.1^{d}	1
Manganese	34.6 ± 0.6^{d}	32.0 ± 0.5^{c}	17.4 ± 0.4^{a}	19.4 ± 0.5^{b}	0.3
Selenium	0.6 ± 0.1^{ns}	0.5 ± 0.2	<DL[2]	0.6 ± 0.2	0.3
Zinc	25.4 ± 0.3^{b}	11.5 ± 0.3^{a}	10.9 ± 0.2^{a}	11.0 ± 0.4^{a}	0.1
Heavy metals (ppm)[3]					
Arsenic	0.4 ± 0.0^{ns}	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.2
Cadmium	<DL	<DL	<DL	<DL	0.05
Lead	<DL	<DL	<DL	<DL	0.3
Mercury	0.008 ± 0.001^{b}	0.007 ± 0.001^{b}	0.003 ± 0.000^{a}	0.004 ± 0.001^{a}	0.003

[1] Values within the same column having different superscript letters are significantly different ($P < 0.05$).
[2] Detection limit.
[3] IUPAC limits according to Becker [1] = arsenic (<2.0), cadmium (<1.0), lead (<5.0) and mercury (<0.1).

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

Table 10
Elemental composition of lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada (n = 3; DW basis)[1].

	Chlorella vulgaris	*Micractinium reisseri*	*Nannochloris bacillaris*	*Tetracystis* sp.	DL[2] (μg g^{-1})
Minerals (g 100 g DW^{-1})					
Calcium	0.49 ± 0.04^{c}	0.42 ± 0.00^{b}	0.26 ± 0.03^{a}	0.36 ± 0.02^{b}	2
Magnesium	0.11 ± 0.00^{c}	0.09 ± 0.00^{b}	0.08 ± 0.01^{b}	0.05 ± 0.00^{a}	1
Phosphorous	0.32 ± 0.01^{a}	0.35 ± 0.00^{a}	0.40 ± 0.03^{b}	0.40 ± 0.00^{b}	0.5
Potassium	0.45 ± 0.01^{a}	0.53 ± 0.00^{b}	0.55 ± 0.05^{b}	0.53 ± 0.00^{b}	5
Sodium	0.11 ± 0.00^{a}	0.11 ± 0.00^{a}	0.13 ± 0.01^{b}	0.14 ± 0.00^{b}	1
Ca:P ratio	1.54 ± 0.11^{d}	1.18 ± 0.01^{c}	0.65 ± 0.02^{a}	0.88 ± 0.04^{b}	–
Trace elements (mg kg DW^{-1})					
Copper	32.5 ± 1.4^{bc}	22.5 ± 0.4^{a}	35.6 ± 3.3^{c}	28.6 ± 0.2^{b}	0.05
Iron	264.2 ± 3.6^{a}	346.3 ± 4.7^{a}	1312.2 ± 153.1^{b}	1616.2 ± 6.1^{c}	1
Manganese	43.8 ± 0.7^{d}	40.4 ± 0.4^{c}	27.5 ± 2.4^{a}	33.0 ± 0.2^{b}	0.3
Selenium	<DL	0.6 ± 0.2^{ns}	<DL	0.6 ± 0.0	0.3
Zinc	35.4 ± 0.6^{c}	18.1 ± 0.4^{a}	20.0 ± 1.5^{a}	23.4 ± 0.4^{b}	0.1
Heavy metals (ppm)[3]					
Arsenic	0.5 ± 0.1^{ns}	<DL	0.4 ± 0.0	<DL	0.2
Cadmium	<DL	<DL	<DL	<DL	0.05
Lead	<DL	<DL	0.4 ± 0.0	<DL	0.3
Mercury	0.006 ± 0.000^{b}	0.006 ± 0.000^{b}	0.005 ± 0.001^{a}	0.005 ± 0.000^{a}	0.003

[1] Values within the same column having different superscript letters are significantly different ($P < 0.05$).
[2] Detection limit.
[3] IUPAC limits according to Becker [1] = arsenic (<2.0), cadmium (<1.0), lead (<5.0) and mercury (<0.1).

in a similar range (0.4–0.5 g cm^{-3}). As such, algal biomass produced from these species and processed in a similar manner is not likely to present any serious logistical challenges if incorporated into conventional feed distribution, storage, processing and delivery systems.

Reported major proximate constituents of algal biomass show highly variable ranges of ash (4–43% DW), protein (6–71%), lipid (1–70%) and carbohydrate (4–64%) which are closely related to variable culture conditions and harvest times [1,35,36]. Additionally, since algal biomass characterization is still in its infancy, nutrient specification standards common for other commercial feed ingredients are not yet established and analytical methods are far from standardized and often inadequate for microalgae [23,49]. As such, projects aimed at defining the nutritional value of specific algal isolates cultured under defined environmental conditions are fool-hardy to rely on historical literature values as a basis for comprehensive nutritional evaluation and formulation of test diets. With regard to gross biochemical composition, ash levels of all biomass in this study were low (<3 g 100 g DW^{-1}); which is generally the case for most freshwater species. Although some dietary ash is important in terms of the essential minerals and trace elements it provides (discussed later), the ash content of livestock, pet food and aquaculture feeds is generally kept to a minimum (<10% of the diet) because of the negative health consequences it can cause [41,50]. Although statistical differences ($P < 0.001$) were observed in ash content between species in this study, it is a result of high repeatability between analytical replicates and the narrow range (1.8–2.8 g 100 g DW^{-1}) is likely of little practical importance. These levels are low and, even if incorporated at relatively high dietary inclusion levels, are not likely to cause adverse health effects or mineral-related toxicities. Typical of microalgae, macromolecular components (e.g., protein, esterifiable lipid and carbohydrate [CHO]) were the major constituents (72–83% DW) and differences between species were small. Whole algal biomass (WAB) from the species studied here contained on average 14 g protein (range, 13–15), 35 g esterifiable lipid (range, 32–36) and 29 g CHO (range, 27–30) per 100 g of biomass DW. Since the cultures were harvested 6 days into stationary phase it is not surprising that protein content is relatively low compared to esterifiable lipid and CHO. If the purpose is to produce large quantities of oil or CHO for bio-energies purposes (e.g., biodiesel, bioethanol); the trade-off will be lower protein production. Since protein typically has a higher market value than lipid and CHO, biorefineries will surely need to exploit residual protein for other purposes (e.g., animal feeds, biomethane, and bioadhesives) in order to increase the economic viability of costly algal biofuel production. As a result, economic models must be developed in order to create cultivation/harvest protocols that effectively balance the relative accumulations of the various organic constituents in a manner that optimized the value proposition of the biomass.

Laboratory and industrial-scale oil extraction methods are rarely ever fully effective. This issue is further complicated for algal biomass where standard extraction procedures are not well established [49,51]. In particular, organic/aqueous solvent extraction of algal biomass using a Soxhlet reactor almost always leaves residual oil in the biomass to varying degrees, especially in the absence of a chemical and/or enzymatic cell-lysis step [37] and this was clearly evident in the present study. The species studied here were mechanically pulverized (Retsch mill) and then 'de-fatted' by solvent extraction under identical conditions ($CHCl_3$:CH_3OH [2:1 v/v], 150 °C, 82 min) [22] and the difference in their lipid-releasing characteristics was profound. Oil fractionation was relatively ineffective for *C. vulgaris* and *M. reisseri* (9–14% recovery) while oil was effectively extracted from *N. bacillaris* and *Tetracystis* sp. (74–83% recovery) and this is likely related to differences in cell wall rigidity and/or porosity between species [37]. This characteristic of *N. bacillaris* and *Tetracystis* sp. makes them quite attractive for both renewable energy and nutrition applications. Downstream processing is one of the most expensive activities associated with transforming raw biomass (including algae) into marketable finished products [50,52]. If new microalgae species are identified that have similar impressive growth rates, productivities and gross biochemical composition to commercially-established species but possess more favorable fractionation characteristics, these species could offer tremendous logistical and nutritional advantages over industrialized species, such as *Chlorella* that requires aggressive and costly mechanical cell disruption processing. Of the species studied here, *Chlorella* is the only one widely used as a dietary supplement for livestock and aquaculture feeds and its success has been impressive with industrial production by >70 companies, generating revenues of several billion dollars annually [53,54].

With regard to mass balance, it is rare that the summation of proximate constituents of algae equals 100% and a wide range of 63–117% has been reported [35]. In the present study, the summation of proximate constituents (including carotenoids) accounted for an average of 85% of total DW (range 81–91%). This finding is generally consistent with Laurens et al. [23] who reported summations of 82–88% using similar methods. The residual may be composed of unaccounted chlorophyll, non-protein nitrogen and vitamins. Some discussion on the estimation of protein content of algal biomass is warranted as it has been

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

reported using different methods of questionable reliability; resulting in published data that is generally unreliable. The two major methods predominantly reported in the literature are spectrophotometric analysis (e.g., Lowry and Bradford) and elemental nitrogen (N) analysis. The method used in this study involving elemental analysis of total N and the use of an 'appropriate' N-to-P conversion factor (e.g., N × 4.78) [27] is the most suitable method and we encourage others to adopt this approach in an effort to standardize protein content estimations of algal biomass. This approach is fully destructive so prior protein extraction steps (e.g., cell wall disruption) are not required and it also eliminates interferences caused by carotenoids and chlorophyll that can make spectrophotometric methods difficult to reproduce. In addition, comparison of protein content data with literature values has been difficult because most authors using N analysis (e.g., Dumas or Kjeldahl methods) have indiscriminately and erroneously employed the N-to-P conversion factor of N × 6.25 to microalgae even after being proven incorrect [56,57]. This N-to-P factor overlooks the assumption that the protein source contains 16% N and does not adequately take into account the often high content of non-protein nitrogen (NPN) found in microalgae. The NPN content of microalgae has been reported to be 4 to 40% depending upon species, season and growth phase [1,27,58]. As such, some of the most comprehensive and commonly cited summaries of protein contents of microalgae and other single-celled proteins (both for research and for product labeling) are likely over-estimates. Ultimately, the most accurate method to estimate protein content is by comprehensive analysis of amino acid profile; however, this may be cost-prohibitive and beyond the requirement for some research and industrial applications. As such, having a highly reliable N-to-P factor for algal biomass that can be applied with relative confidence to a wide range of species and cultivation/harvest conditions is of great value. In this study, we tested the utility of the recommended N-to-P factor for algal biomass (N × 4.78) [27] by making a comparison of the protein content results with those estimated using species-specific N-to-P factors (N × k) calculated for each species based on their individual amino acid contributions [28]. We found close agreement (<2.5%, Fig. 1) between the two methods with an average deviation of only 1.2%. Consequently, we suggest that in the absence of costly comprehensive analyses of amino acid profiles, the N-to-P factor of N × 4.78 can be confidently used as a reasonable estimation of protein content of algal biomass. This finding provides further evidence that the N-to-P factor of N × 6.25 which has been historically applied is incorrect and it should be avoided [1,23,27].

As previously discussed total CHO levels of WAB were in a narrow range (27–30 g 100 g DW^{-1}). Interestingly, the composition of that CHO was dramatically different between *C. vulgaris* and *M. reisseri* and *N. bacillaris* and *Tetracystis* sp. The former species being good at producing starch (52–64% of total CHO) while the latter accumulated very little starch (~5% of total CHO). While all microalgae produce hydrocarbons as energy and carbon stores, some groups of microalgae have a preference for starch rather than lipid accumulation and these species are gaining attention as potential feedstocks for bioethanol production [59]. The fact that *C. vulgaris* biomass used in this study contained CHO composed of a roughly equal mixture of both starch (52% of total CHO) and fiber (48% of total CHO) is in agreement with Matsumoto et al. [60]. These authors reported that *Chlorella* sp. (and similar species like *Scenedesmus*, *Chlamydomonas* and *Tetraselmis*) typically produces large amounts of both starch (as energy and carbon reserves) and fibrous cellulose (for cell wall structure). It is important to also note that, of the fiber fraction, generally comprised of cellulose, hemicellulose and lignin in most terrestrial plants, microalgae fiber contains no lignin and low hemicellulose levels. As a result, it has been proposed that the use of starch-rich algal biomass as feedstock for bioethanol production may be advantageous over conventional feedstocks by providing increased hydrolysis efficiency, higher fermentable yields and reduced production costs [60]. *M. reisseri*, being so similar in other aspects of its growth performance and biochemical composition, it is not surprising to see similar CHO compositions of starch (64% of total CHO) and fiber (36% of total CHO). Fiber has low digestibility in monogastric animals, whereas starch can be a readily available and inexpensive source of digestible energy [38,39]. Since it appears that the composition of algal starch is similar to that of cereal grains commonly used in animal nutrition at ~34% amylose and 66% amylopectin [61], *C. vulgaris* and *M. reisseri* biomass could be of interest, not only as feedstocks for bioethanol, but also directly as an inexpensive source of digestible energy for monogastric animal feeds. Alternatively, coupling of several activities together most likely holds the most promise for economically-sustainable biomass valorization. In the same manner that grains, corn and oilseeds are fractionated to isolate lipids (for biodiesel and/or edible oils) and fermentable carbohydrates (for bioethanol), the protein residues in the 'cake' become naturally concentrated to higher levels (30–75% DW) with substantially lower levels of poorly digestible CHOs. These biofuel co-products (e.g., distillers dried grains with solubles [DDGS], high protein distillers dried grains [HPDDG] and corn protein concentrate [CPC]) are marketed predominantly for animal and fish feeds and represent the major revenue stream for biofuel and bioproduct companies [62]. Given the high protein productivity and protein quality of some microalgae, the developing algal biofuel sector would surely benefit by adopting this strategy by producing and marketing amino acid-rich algal protein concentrates (APCs) to off-set the high costs associated with industrial algal production; especially if coupled with a bioremediation strategy [63,64]. A review of these biorefinery processes and their application for renewable energy and animal feeds can be found in Makkar [65]. Given that the majority (>95%) of total CHO found in *N. bacillaris* and *Tetracystis* sp. biomass was fiber, use of these species in animal nutrition would be better suited to ruminant, as opposed to monogastric, animal feeds because of ruminant animals' high capacity to digest the cellulosic cell wall material. Since the cultures in this study were cultivated under the same conditions, harvested at the same growth phase and had similar contents of ash (2 g 100 g DW^{-1}), protein (14 g 100 g DW^{-1}), esterifiable lipid (32–36 g 100 g DW^{-1}) and total CHO

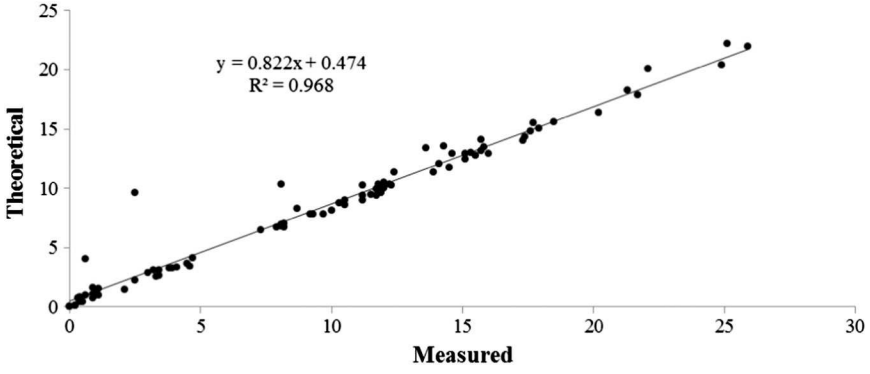

Fig. 1. Regression of measured and theoretical AA compositions (mg g DW^{-1}) of lipid-extracted biomass produced from microalgae species isolated from freshwater in Alberta, Canada.

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

(27–30 g 100 g DW^{-1}), it is possible that there is a true genetic difference between *C. vulgaris* and *M. reisseri* and *N. bacillaris* and *Tetracystis* sp. in their propensity to produce starch as a storage reserve. However, further studies would be required to form consensus as it is has also been demonstrated that various algal species can have a differential response to environmental factors known to influence starch synthesis and degradation; such as light intensity and spectrum, temperature and nutrient limitation [66]. Although not quantified in the present study, *C. vulgaris* starch reserves (and possibly that of *M. reisseri*) contains β-1,3-glucan which has been shown to stimulate the immune response, fight cancer and promote infectious disease resistance [54]. This property has the potential to greatly increase the economic value of algal biomass as a functional ingredient for animal feeds.

Algal oil is a potential source digestible energy (e.g., MUFAs) and high-value essential fatty acids (e.g., PUFAs) for animal feed applications [1]. In the present study, the esterifiable lipid fraction of all biomass was comprised predominantly of oleic acid (18:1n − 9; 33–47% of total FA), was rich in highly digestible MUFA (average 47%; range 40–53%) and PUFA (average 32%; range 27–40%) and low in SFAs (average 19%; range 14–24%). In comparison to other oil sources used in feed, the sum of SFA, MUFA and PUFA were similar with peanut oil (MUFA 46%, PUFA 32% and SFA 18%) [39]. Although PUFA levels were relatively high for all species (>25% of total FA), it was composed of medium-chain PUFA (e.g., C_{16} and C_{18}) and devoid of long-chain (LC) PUFA (e.g., C_{20} and C_{22}). This is generally typical for freshwater microalgae and make them poor sources of nutritionally-essential LC-PUFA, namely arachidonic acid (ARA; 20:4n − 6), eicosapentaenoic acid (EPA; 20:5n − 3) and docosahexaenoic acid (DHA; 22:6n − 3). For this reason, *N. bacillaris* and *Tetracystis* sp. are more interesting for nutrition applications than *C. vulgaris* and *M. reisseri* due to their significantly higher levels (9–12 vs 5–7% of total FA) of α-linolenic acid (ALA; 18:3n − 3) which is the dietary precursor for cellular biosynthesis of EPA and DHA [67]. In addition, due to significantly lower levels (13 vs 21–25% of total FA) of linoleic acid (18:2n − 6), *N. bacillaris* and *Tetracystis* sp. also have higher n − 3:n − 6 ratios (0.5–0.7:1) than *C. vulgaris* and *M. reisseri* (0.2–0.3:1) and are similar to that of canola oil (0.6:1). Although the n − 3:n − 6 ratios of these algal species cannot compete with fish oils (3–24:1) and certain high n − 3 terrestrial plant oils like flax and echium oils (2–4:1), they are substantially higher than those found in most vegetable oils produced from conventional crops (0–0.1:1) and rendered animal fats (0–0.2:1) commonly used in animal feeds [39]. Since algal biomass with relatively low protein and high CHO would likely compete with conventional plant-based 'flours' rather than high-protein fish meals, this higher n − 3:n − 6 ratio could provide a competitive advantage. Although dietary fatty acid requirements and optimum n − 3:n − 6 ratios vary between farmed animal species; generally diets that fall below a ratio of 1:1 have been implicated with disease pathogenesis such as cardiovascular disease, cancer and impaired immune function while higher ratios promote an anti-inflammatory response in humans and animals resulting in improved cardiac and nervous system health and increased cell membrane fluidity [68]. Since animals lack the ability to convert dietary linoleic acid (LA; 18:2n − 6) into α-linolenic acid (ALA; 18:3n − 3), the cellular fatty acid biosynthesis pathways shifts towards overproduction of ARA (20:4n − 6) when n − 3:n − 6 ratios are low [67]. Under this situation, since ARA and EPA compete for the same lipoxygenase or cyclooxygenase enzyme, a greater proportion of high activity eicosanoids (inflammatory) are produced than low activity eicosanoids (anti-inflammatory) and animal health is compromised. Since livestock and fish diets are composed of mixtures of feed ingredients high in terrestrial plant sources (even for carnivorous species), supplementation with similar algal products/co-products has the potential to off-set dietary fatty acid imbalances in addition to providing a source of digestible energy and other essential nutrients.

For animal feeding applications, protein generally has higher economic value than other major nutrients (e.g., lipid and CHO). As such, the feed sector is continually seeking new economical, nutritious, functional and sustainable protein sources to incorporate into dietary formulations [12]. Although dietary protein requirements have been established for most farmed species at various life stages [38,39,50], animals do not have a true dietary requirement for protein per se. Rather, they require the building blocks that make up that protein (e.g., amino acids, AAs) and, in particular, roughly 10 essential AAs. These EAAs are generally the same for all animal species (including humans) and are those that the animal cannot synthesize endogenously or cannot synthesize in sufficient quantities to support maximum growth rate and/or productivity. The ration which has the highest protein quality is typically the one which supplies all of the EAAs needed in proportions most similar to those in which they exist in the protein (products) to be formed (e.g., muscle, milk, and eggs), plus an appropriate non-specific source of nitrogen to form the non-EAAs [45]. As mentioned, commercial feeds for livestock and fish are comprised of mixtures of feed ingredients high in terrestrial plant sources such as corn, soybean, canola and wheat. These ingredients are generally deficient in certain EAAs (usually lysine, methionine and tryptophan) so formulated diets are routinely supplemented with costly chemically-synthesized crystalline AAs to correct for this. Studies have shown that algal biomass derived from certain commercialized species (e.g., *Chlorella*, *Spirulina*) generally contain all the EAAs in proportions more suited to animal nutrition than many of these terrestrial plant-based crops [1,56]. In the present study, we demonstrate that for the majority of the EAAs (7 of 10), their levels found in the biomass are generally higher than soybean protein which has one of the best EAA balances of all the conventional plant-based crops. Lysine, in particular, is generally the first-limiting EAA in animal feeds and its levels in the algal biomass studied here (6.0–8.6 g 100 g $protein^{-1}$) is generally higher than those of soybean protein (6.4 g 100 g $protein^{-1}$), *Spirulina* (4.2–7.7 g 100 g $protein^{-1}$) and the reference protein egg albumin (5.3 g 100 g $protein^{-1}$). Lysine concentrations observed in the present study represent 0.9–1.5 g lysine 100 g DW^{-1} and greatly exceed those found in comparable carbohydrate-rich feed ingredients currently used in animal nutrition such as corn (0.2–1.1 g lysine 100 g DW^{-1}), wheat (0.1–0.7 g lysine 100 g DW^{-1}) and distillers dried grains (0.1–0.9 g lysine 100 g DW^{-1}) [39].

In the absence of more rigorous in vitro and/or in vivo nutritional evaluations, EAA indices provide good approximations of protein quality [26]. Algal products/co-products are said to be of high protein quality if they have an EAA indices of >0.90, moderate quality when 0.70–0.89 and low quality when <0.70 [69]. As such, the protein quality of all WAB and most LEB (3 out of 4) produced for this study was high (0.90–1.10) or moderate for one LEB sample (0.86). The causative agent for this lower indices will be discussed in the following section. EAA indices for algal biomass are rarely found in the published literature and have not been reported for most of the species studied here (with exception of *Chlorella*). The range we observed (0.86–1.10) agrees well with Brown and Jeffrey [69] and Tibbetts et al. [70,71] who also reported moderate to high protein quality of other algal species (0.80–1.25). While EAA indices of biomass from *C. vulgaris* (0.92–0.93), *M. reisseri* (0.90–0.92) and *N. bacillaris* (0.86–1.07) were similar or exceeded that of soybean protein (0.9), *Tetracystis* sp. had EAA indices (WAB, 1.10; LEB, 1.02) exceeding those of the ideally-balanced EAA profile of reference protein egg albumin (1.0) [57]. As such, supplementation of formulated feeds with similar algal products/co-products has the potential to off-set dietary EAA imbalances and to minimize the use of costly chemically-synthesized crystalline AAs.

Down-stream processing has the potential to alter the nutritional value of algal products/co-products, so, although a full evaluation is beyond the scope of this study, some discussion is warranted. While extraction of oil from biomass effectively concentrates total protein levels in the residual 'cake', the individual AAs that make up that protein may be affected differentially. The extents of the effects are dependent upon the method of extraction, the processing conditions under which they

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

are operated and the individual stability of particular AAs [72,73]. In most cases, AAs in conventional raw biomass are readily stable under industrial processing conditions and become concentrated to higher levels in the co-products, and this was generally the case here. AA contents of the WAB were increased in the LEB by 17–23% for *C. vulgaris* and *M. reisseri* and 42–59% for *N. bacillaris* and *Tetracystis* sp.; reflecting varying lipid-extraction efficiencies. However, these are average values and do not reflect the 'conservation' of specific AAs in the biomass. At the same time that AAs become concentrated by oil removal, some may be lost to varying degrees by the lipid-extraction process itself. To better understand which (if any) specific AAs were destroyed by processing, we calculated 'theoretical' AA levels in the LEB; based on the known percentage of oil removal from the WAB; assuming an ideal situation of no AA destruction. By contrasting these theoretical with measured AA concentrations in the LEB, it was possible to: 1) assess the overall AA loss due to processing and 2) expose specific AAs that were highly impacted by the processing. Overall, AA conservation was very high as demonstrated by high correlation between measured and theoretical AA concentrations in the LEB of each species: *C. vulgaris* ($r = 0.999$; $P < 0.001$), *M. reisseri* ($r = 0.997$; $P < 0.001$), *N. bacillaris* ($r = 0.968$; $P < 0.001$) and *Tetracystis* sp. ($r = 0.991$; $P < 0.001$). This result provides evidence that AA destruction was minimal under the particular processing conditions used in this study ($CHCl_3$:CH_3OH [2:1 v/v], 150 °C, 82 min). In addition, by regressing the measured and theoretical AA concentrations of the pooled data set (Fig. 1), it is clear that they correlate well ({Theoretical = [0.822 × Measured] + 0.474}; $R^2 = 0.968$) with an average variance of only 1.2 mg g DW^{-1}. However, it is evident that levels of the EAAs methionine and tryptophan were compromised in *N. bacillaris*. While these EAAs were concentrated and conserved in the LEB of the other species, *N. bacillaris* lost 85 and 74% of its original methionine and tryptophan, respectively. From the previous section it was clear that, even before further examination, that the EAAs of *C. vulgaris*, *M. reisseri* and *Tetracystis* sp. were likely largely conserved during processing because EAA indices of their WAB (0.93, 0.92 and 1.10, respectively) were close (<7% variation) to those of their LEB (0.92, 0.90 and 1.02, respectively); also evidenced by the high precision of the linear regression model above. As for *N. bacillaris*, however, a large (20%) decrease was observed in the EAA indices from its WAB (1.07) to its LEB (0.86) and this is a result of methionine and tryptophan loss, as discussed.

There is limited data available on the elemental composition of microalgae biomass; which is in contrast to macroalgae (seaweed) biomass where numerous species have been well characterized [74]. This is not entirely unexpected because, although microalgae may contain certain elements of interest for nutrition, the inorganic elemental composition (ash) of microalgae (excluding diatoms) is generally much lower (4–20%) than macroalgae (22–64%) [35]. In general, the algal biomass studied is not particularly unique in its mineral and trace element composition relative to other commonly-used ingredients for animal feeding; with exception of their Fe content and Ca:P ratio. Relative to similar terrestrial carbohydrate-rich flours like brewers dried grains and wheat by-products, biomass from *N. bacillaris* and *Tetracystis* sp. were particularly rich sources of Fe (800–1616 mg kg DW^{-1}) with attractively low Ca:P ratios (0.6–0.9:1). Fe is an essential trace element in animal and fish nutrition and is largely involved in cellular respiration, oxygen transport, acid–base balance and energy metabolism. As such, adequate Fe levels are required in the diet as it forms a vital component of red blood cell (erythrocyte) hemoglobin and plasma-transported circulatory system enzymes and, when inadequate, conditions of anemia generally occur. Fe requirements vary between species, life stage and nutritional status but are always among the highest of all the essential trace elements at 30–40 mg kg $diet^{-1}$ for ruminant livestock, 40–80 mg kg $diet^{-1}$ for monogastric livestock and 20–180 mg kg $diet^{-1}$ for farmed fish [39,50]. The high levels of Fe observed in this study for *N. bacillaris* and *Tetracystis* sp. biomass are consistent with reports for commercially-established algal species like *Spirulina* and may provide a unique and highly-marketable property for certain algal products/co-products. From an animal health perspective, new data has recently emerged that indicates that feeding diets supplemented with Fe-rich algal biomass to pigs and rats significantly increased erythrocyte volumes and blood hemoglobin concentrations [75–77] and this area warrants further investigation. Ca and P are the most critical essential minerals involved in the development and maintenance of the skeletal system and maintaining acid–base homeostasis in farmed animals and fish [39,50]. Complexed together, they constitute the principle component of animal bone (hydroxyapatite; $(Ca_5(PO_4)_3(OH))$) so, not only their individual dietary concentrations, but also their relative proportions to one another can influence their bioavailability, metabolism and physiological utilization in the animal. The dietary Ca:P ratio recommended as most suitable for young rapidly growing farmed livestock and fish is <2:1 and optimally 1:1; with the exception of mature production animals (e.g., layer chickens, dairy cattle) which may require much higher ratios (e.g., 12:1) to support egg shell development and milk production [38,39,50]. A deficiency in either mineral or a substantial imbalance in the dietary Ca:P ratio (especially if compounded with low dietary vitamin D) generally results in depressed growth, poor feed conversion and/or production efficiency, anorexia and, in severe cases, skeletal malformations (e.g., rickets, osteomalacia). The well-balanced Ca:P ratios observed in this study (average 1:1, range 0.6–1.5) are consistent with reports for commercially-established algal species like *Spirulina* (1.0–1.7:1), which are both better balanced than some other microalgae species (e.g., *Tetraselmis*, *Dunaliella*, 2.9–3.2:1), although cultivation/harvest conditions can highly influence these values. These attractive Ca:P ratios may provide another unique avenue for certain algal products/co-products; even if as an indirect benefit of algal supplementation for other nutritional and/or functional purposes (e.g., EAA or Fe supplementation) since it is not likely that algal biomass would be used solely as a mineral supplement. All measured heavy metal concentrations (e.g., As, Cd, Pb, Hg) in this study were several magnitudes lower than proposed upper limits for safe consumption as animal feeds [1]. However, if similar algal biomass is produced utilizing industrial waste streams (e.g., wastewater, flue gas) this may not be the case and must be a priority consideration for its nutritional and safety evaluation.

Given their relatively low protein and high CHO levels, all biomass produced for this study would be considered protein-rich algal 'flours' with a market value similar to that of wheat and other cereal grain by-products (<$500 t^{-1}) as opposed to higher value protein-rich algal 'meals' such as those produced from terrestrial oilseed crops (e.g., soy, canola, corn). For this reason, these products are more likely to find a place in relatively inexpensive ruminant animal feeds (e.g., cattle, sheep, deer) rather than higher value monogastric animal feeds (e.g., poultry, swine, fish). In addition, the high residual esterifiable lipid content of *C. vulgaris* and *M. reisseri* represent inefficiency for biofuel applications and could cause logistical problems in feed processing systems for animal feed applications. However, since the fatty acid profile of that lipid is nutritionally attractive, these products could be marketed as 'high-oil residue meals' which has the potential to raise their economic value although unique markets and appropriate storage and processing conditions would need to be identified. Additionally, ruminant animals are better equipped to digest the algal cell wall material in these relatively unprocessed products than monogastric animals [55]. Several avenues exist that could increase their economic value. Modified cultivation/harvesting protocols could easily be employed to enhance protein accumulation and/or the biomass could be 'upgraded' to produce protein concentrates, which is a common practice in the animal feed sector. Additionally, if it can be demonstrated that algal products have 'functional ingredient' properties (e.g., high response at low dose) their economic value may be considerably increased; provided that significant production tonnage can be economically achieved. These types of ingredients commonly referred to as 'GEMs' (Gut Environment Modifiers) are highly sought after for animal feed applications. In general, GEMs provide prebiotic or probiotic properties, enhanced digestion, metabolism and

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

physiological utilization of dietary nutrients or provide certain trace nutrients (limiting amino acids, essential trace elements, etc.) that may promote positive animal health, immunostimulatory effects and disease resistance. In vitro investigations are currently underway to evaluate these products for nutrient digestibility, metabolizable energy content, ruminal gas (CO_2, CH_4 and NH_3–N) and volatile fatty acid production and assessment for potential consumption risk.

Acknowledgments

The authors wish to thank Kathryn Dickinson for valuable advice and technical expertise and Dr. Kelly Soanes for reviewing a draft of this manuscript. This work was supported by the NRCC Algal Carbon Conversion (ACC) program and is NRCC publication no. 56096.

References

[1] E.W. Becker, Microalgae for human and animal nutrition, in: A. Richmond, Q. Hu (Eds.), Handbook of Microalgal Culture: Applied Phycology and Biotechnology, Second ed.John Wiley and Sons, Ltd., Blackwell Publishing Ltd., United Kingdom, 2013, pp. 461–503.
[2] T.M. Mata, A.A. Martins, N.S. Caetano, Microalgae for biodiesel production and other applications: a review, Renew. Sustain. Energy Rev. 14 (2010) 217–232.
[3] T. Lundquist, I. Woertz, N. Quinn, J. Benemann, A Realistic Technology and Engineering Assessment of Algae Biofuel Production, Energy Biosciences Institute, University of California-Berkeley, 2010. (November 2010, http://www.energybiosciencesinstitute.org/media/AlgaeReportFINAL.pdf (accessed August 26, 2014)).
[4] L. Soh, M. Montazeri, B.Z. Haznedaroglu, C. Kelly, J. Peccia, M.J. Eckelman, J.B. Zimmerman, Evaluating microalgal integrated biorefinery schemes: empirical controlled growth studies and life cycle assessment, Bioresour. Technol. 151 (2013) 19–27.
[5] K. Soratana, W.J. Barr, A.E. Landis, Effects of co-products on the life-cycle impacts of microalgal biodiesel, Bioresour. Technol. 159 (2014) 157–166.
[6] J.J. Cheng, G.R. Timilsina, Status and barriers of advanced biofuel technologies: a review, Renew. Energy 36 (2011) 3541–3549.
[7] B. Subhadra, G. Grinson, Algal biorefinery-based industry: an approach to address fuel and food insecurity for a carbon-smart world, J. Sci. Food Agric. 91 (2011) 2–13.
[8] F. Ahmed, Y. Li, P.M. Schenk, Algal biorefinery: sustainable production of biofuels and aquaculture feed? in: R. Gordon, J. Seckbach (Eds.), The Science of Algal Fuels — Phycology, Geology, Biophotonics, Genomics and Nanotechnology, Springer Science, New York, 2012, pp. 23–41.
[9] M. Vanthoor-Koopmans, R.H. Wijffels, M.J. Barbosa, M.H.M. Eppink, Biorefinery of microalgae for food and fuel, Bioresour. Technol. 135 (2013) 142–149.
[10] D. Brune, T. Lundquist, J. Benemann, Microalgal biomass for greenhouse gas reductions: potential for replacement of fossil fuels and animal feeds, J. Environ. Eng. 135 (2009) 1136–1144.
[11] R. Harun, M. Singh, G.M. Forde, M.K. Danquah, Bioprocess engineering of microalgae to produce a variety of consumer products, Renew. Sustain. Energy Rev. 14 (2010) 1037–1047.
[12] Micro Market Monitor, Global feed market research report, http://www.micromarketmonitor.com (accessed August 28, 2014).
[13] K. Hudek, L.C. Davis, J. Ibbini, L. Erickson, Commercial products from algae, in: R. Bajpai, A. Prokop, M. Zappi (Eds.), Algal Biorefineries, Springer Science, New York, 2014, pp. 275–295.
[14] J. Benemann, Microalgae for biofuels and animal feeds, Energies 6 (2013) 5869–5886.
[15] J.Y. Lee, S.Y. Jun, C.Y. Ahn, H.M. Oh, Comparison of several methods for effective lipid extraction from microalgae, Bioresour. Technol. 101 (2010) 71–74.
[16] V. Draganovic, S.E. Jørgensen, R. Boom, J. Jonkers, G. Riesen, A.J. van der Goot, Sustainability assessment of salmonid feed using energy, classical exergy and eco-exergy analysis, Ecol. Indic. 34 (2013) 277–289.
[17] S. Gatrell, K. Lum, J. Kim, X.G. Lei, Potential of defatted microalgae from the biofuel industry as an ingredient to replace corn and soybean meal in swine and poultry diets, J. Anim. Sci. 92 (2014) 1306–1314.
[18] S. Bhatti, M.J. MacPherson, C. Kozera, P.J. McGinn, S.J.B. O'Leary, Microalgae conversion of industrial carbon dioxide emissions to value-added biomass and biofuels: sampling, isolation and screening of microalgal strains for industrial deployment in Canada, Fourth International Conference on Algal Biomass, Biofuels and Bioproducts, June 15–18, 2014, Santa Fe, New Mexico, USA2014 (O.2.37).
[19] K.C. Park, C. Whitney, J.C. McNichol, K.E. Dickinson, S. MacQuarrie, B.P. Skrupski, J. Zou, K.E. Wilson, S.J.B. O'Leary, P.J. McGinn PJ, Mixotrophic and photoautotrophic cultivation of 14 microalgae isolates from Saskatchewan, Canada: potential applications for wastewater remediation for biofuel production, J. Appl. Phycol. 24 (2012) 339–348.
[20] R.A. Anderson, J.A. Berges, P.J. Harrison, M.M. Watanabe, Recipes for freshwater and seawater media, in: R.A. Anderson (Ed.), Algal Culturing Techniques, Elsevier Academic Press, Massachusetts2005 (507 pp.).
[21] S.M. Armstrong, L.S. Staples, A.G. Bauder, J.S. Craigie, Photobioreactor, National Research Council of Canada, 2002, Canadian patent application no. 2394518.
[22] K.M. MacDougall, J. McNichol, P.J. McGinn, S.J.B. O'Leary, J.E. Melanson, Triacylglycerol profiling of microalgae strains for biofuel feedstock by liquid chromatography–high-resolution mass spectrometry, Anal. Bioanal. Chem. 401 (2011) 2609–2616.
[23] L.M. Laurens, T.A. Dempster, H.D.T. Jones, E.J. Wolfrum, S. Van Wychen, J.S.P. McAllister, M. Rencenberger, K.J. Parchert, L.M. Gloe, Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries, Anal. Chem. 84 (2012) 1879–1887.
[24] R.L. Heinriksen, S.C. Meredith, Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate, Anal. Biochem. 136 (1984) 65–74.
[25] J.A. White, R.J. Hart, J.C. Fry, An evaluation of the Waters Pico-Tag system for the amino-acid analysis of food materials, J. Clin. Lab. Autom. 8 (1986) 170–177.
[26] B.L. Oser, Method for integrating essential amino acid content in the nutritional evaluation of protein, J. Am. Diet. Assoc. 27 (1951) 396–402.
[27] S.O. Lourenço, E. Barbarino, P.L. Lavín, U.M.L. Marquez, E. Aidar, Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors, Eur. J. Phycol. 39 (2004) 17–32.
[28] J. Mossé, Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed protein content, J. Agric. Food Chem. 38 (1990) 18–24.
[29] P.J. McGinn, K.E. Dickinson, K.C. Park, C.G. Whitney, S.P. MacQuarrie, F.J. Black, J. Frigon, S.R. Guio, S.J.B. O'Leary, Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus* sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode, Algal Res. 1 (2012) 155–165.
[30] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[31] A. Sukenik, O. Zmora, Y. Carmeli, Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II. *Nannochloropsis* sp. Aquaculture 117 (1993) 313–326.
[32] B. Fernandes, G. Dragone, A.P. Abreu, P. Geada, J. Teixeira, A. Vicente, Starch determination in *Chlorella vulgaris* — a comparison between acid and enzymatic methods, J. Appl. Phycol. 24 (2012) 1203–1208.
[33] Environmental Protection Agency, Test Methods for Evaluating Solid Waste Physical/Chemical Methods, Methods EPA 6010C and 7471B, 2007.
[34] V.H. Smith, Progress in algae as a feedstock for bioproducts, Ind. Biotechnol. 10 (2014) 159–161.
[35] J.K. Volkman, M.R. Brown, Nutritional value of microalgae and applications, in: D.V. Subba Rao (Ed.), Algal Cultures, Analogues of Blooms and Applications, CABI Publishing, 2009, pp. 407–457.
[36] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294–306.
[37] L.M. Laurens, M. Quinn, W.S. Van, D.W. Templeton, E.J. Wolf, Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by *in situ* transesterification, Anal. Bioanal. Chem. 403 (2012) 167–178.
[38] National Research Council, Nutrient Requirements of Poultry, National Academy Press, Washington, 1994. (155 pp.).
[39] National Research Council, Nutrient Requirements of Fish and Shrimp, National Academy Press, Washington, 2011. (376 pp.).
[40] M.A. Zafra, F. Molina, A. Puerto, The neural/cephalic phase reflexes in the physiology of nutrition, Neurosci. Biobehav. Rev. 30 (2006) 1032–1044.
[41] S.P. Lall, S.M. Tibbetts, Nutrition, feeding, and behaviour of fish, in: L. Hess, N. Antinoff (Eds.),Nutrition and behaviour of uncommon species, Vet. Clin. North Amer. - Exot. Anim. Pract., 12, 2009, pp. 361–372.
[42] A. Ginzberg, M. Cohen, U. Sod-Moriah, S. Shany, A. Rosenshtrauch, S. Arad, Chickens fed with biomass of the red microalga *Porphyridium* sp. have reduced blood cholesterol level and modified fatty acid composition in egg yolk, J. Appl. Phycol. 12 (2000) 325–330.
[43] M.F. Fuller, The Encyclopedia of Farm Animal Nutrition, CABI Publishing, Massachusetts, 2004. 68–69.
[44] E.W. Crampton, L.E. Harris, Applied Animal Nutrition, Second edition W.H. Freeman and Company, San Francisco, 1969. (753 pp.).
[45] L.A. Maynard, J.K. Loosli, H.F. Hintz, R.G. Warner, Animal Nutrition, Seventh edition McGraw-Hill, New York, 1979. (603 pp.).
[46] Y.K. Chang, S.S. Wang, Advances in Extrusion Technology: Aquaculture/Animal Feeds and Foods, Technomic Publishing Company Inc., Pennsylvania, 1999. (422 pp.).
[47] J.W. Hertrampf, F. Piedad-Pascual, Handbook on Ingredients for Aquaculture Feeds, Kluwer Academic Publishers, Boston, 2000. (573 pp.).
[48] N. Letsche, P.J. Lammers, Bulk density of bio-fuel by-products, Iowa State University Animal Industry ReportA.S. Leaflet R24592009. (http://www.ans.iastate.edu/report/air/2009pdf/R2459.pdf (accessed July 24, 2014)).
[49] Y. Li, F.G. Naghdi, S. Garg, T.C. Adarme-Vega, K.J. Thurecht, W.A. Ghafor, S. Tannock, P.M. Schenk, A comparative study: the impact of different lipid extraction methods on current microalgal lipid research, Microb. Cell Fact. 13 (2014) 1–9.
[50] P. McDonald, R.A. Edwards, J.F.D. Greenhalgh, C.A. Morgan, L.A. Sinclair, Animal Nutrition, Seventh edition Prentice Hall, New York, 2010. (692 pp.).
[51] D.C. Kannan, V.M. Pattarkine, Recovery of lipids from algae, in: R. Bajpai, A. Prokop, M. Zappi (Eds.), Algal Biorefineries, Springer Science, New York, 2014, pp. 297–310.
[52] F. Delrue, P.A. Setier, C. Sahut, L. Cournac, A. Roubaud, G. Peltier, A.K. Froment, An economic, sustainability, and energetic model of biodiesel production from microalgae, Bioresour. Technol. 111 (2012) 191–200.
[53] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Review: commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96.
[54] J.A. Kralovec, C.J. Barrow, Immunoenhancing preparations of marine origin, in: C.J. Barrow, F. Shahidi (Eds.), Marine Nutraceuticals and Functional Foods, CRC Press, Boca Raton, 2008, pp. 451–466.
[55] L. Gouveia, A.P. Batista, I. Sousa, A. Raymundo, N.M. Bandarra, Microalgae in novel food products, in: K. Papadoupoulos (Ed.), Food Chemistry Research Developments, Nova Science Publishers, New York, 2008, pp. 75–112.
[56] I.S. Chronakis, Biosolar proteins from aquatic algae, Dev. Food Sci. 41 (2000) 39–75.

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011

[57] E.W. Becker, Micro-algae as a source of protein, Biotechnol. Adv. 25 (2007) 207–210.
[58] C.V. González López, M.C. Cerón García, F.C. Acién Fernández, C.S. Bustos, Y. Chisti, J.M. Fernández Sevilla, Protein measurements of microalgal and cyanobacterial biomass, Bioresour. Technol. 101 (2010) 7587–7591.
[59] K. Li, S. Liu, X. Liu, An overview of algae bioethanol production, Int. J. Energy Res. 38 (2014) 965–977.
[60] M. Matsumoto, H. Yokouchi, N. Suzuki, H. Ohata, T. Matsunaga, Saccharification of marine microalgae using marine bacteria for ethanol production, Appl. Biochem. Biotechnol. 105–108 (2003) 247–254.
[61] B. Maršálková, M. Širmerová, M. Kuřec, T. Bányik, I. Bányiková, K. Melzoch, V. Zachleder, Microalgae *Chlorella* sp. as an alternative source of fermentable sugars, Chem. Eng. Trans. 21 (2010) 1279–1284.
[62] Renewable Fuels Association, 2013 Ethanol Industry Outlook, Renewable Fuels Association, Washington, 2013.
[63] P. McKendry, Energy production from biomass (part 2): conversion technologies, Bioresour. Technol. 83 (2002) 47–54.
[64] J.M.R. García, F.G.A. Fernández, J.M.F. Sevilla, Development of a process for the production of L-amino-acids concentrates from microalgae by enzymatic hydrolysis, Bioresour. Technol. 112 (2012) 164–170.
[65] H.P.S. Makkar, Biofuel Co-products as Livestock Feed — Opportunities and Challenges, Food and Agriculture Organization, Rome, 2012. (533 pp.).
[66] V. Zachleder, I. Bányiková, Starch overproduction by means of algae, in: R. Bajpai, A. Prokop, M. Zappi (Eds.), Algal Biorefineries, Springer Science, New York, 2014, pp. 217–240.
[67] S.M. Hixson, Fish nutrition and current issues in aquaculture: the balance in providing safe and nutritious seafood, in an environmentally sustainable manner, J. Aquacult. Res. Dev. 5 (2014), http://dx.doi.org/10.4172/2155-9546.1000234.
[68] A.P. Simopoulos, The importance of the ratio of omega-6/omega-3 essential fatty acids, Biomed. Pharmacother. 56 (2002) 365–379.
[69] M.R. Brown, S.W. Jeffrey SW, Biochemical composition of microalgae from the green algal classes *Chlorophyceae* and *Prasinophyceae*. 1. Amino acids, sugars and pigments, J. Exp. Mar. Biol. Ecol. 161 (1992) 91–113.
[70] S.M. Tibbetts, J.E. Milley, S.P. Lall, Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors, J. Appl. Phycol. (2014), http://dx.doi.org/10.1007/s10811-014-0428-x.
[71] S.M. Tibbetts, W.J. Bjornsson, P.J. McGinn, Biochemical composition and amino acid profiles of *Nannochloropsis granulata* algal biomass before and after supercritical fluid CO_2 extraction at two processing temperatures, Anim. Feed Sci. Technol. (2014) (submitted for publication).
[72] R. Sarada, M.G. Pillai, G.A. Ravishankar, Phycocyanin from *Spirulina* sp.: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin, Process Biochem. 34 (1999) 795–801.
[73] D.M. Bastos, E. Monaro, E. Siguemoto, M. Séfora, Maillard reaction products in processed food: pros and cons, in: B. Valdez (Ed.), Food Industrial Processes — Methods and Equipment, InTech Open Science, Croatia, 2012, pp. 281–300.
[74] V. Venugopal, Seaweed: nutritional value, bioactive properties, and uses, in: V. Venugopal (Ed.), Marine Products for Healthcare: Functional and Bioactive Nutraceutical Compounds From the Ocean, CRC Press Taylor and Francis Group, Boca Raton, 2009, pp. 261–295.
[75] A.M. Burja, H. Radianingtyas, Nutraceuticals and functional foods from marine microbes: an introduction to a diverse group of natural products isolated from marine macroalgae, microalgae, bacteria, fungi, and cyanobacteria, in: C.J. Barrow, F. Shahidi (Eds.), Marine Nutraceuticals and Functional Foods, CRC Press, Boca Raton, 2008, pp. 367–403.
[76] L.M. Moreira, B. Behling, R.S. Rodrigues, J.A.V. Costa, L.A. de Souza Soares, *Spirulina* as a protein source in the nutritional recovery of Wistar rats, Braz. Arch. Biol. Technol. 56 (2013) 447–456.
[77] J. Kim, X.G. Lei, Potential of a defatted microalgal biomass as an iron source for hemoglobin repletion, FASEB 28 (2014) 828.8.

Please cite this article as: S.M. Tibbetts, et al., Biochemical characterization of microalgal biomass from freshwater species isolated in Alberta, Canada for animal feed applications, Algal Res. (2014), http://dx.doi.org/10.1016/j.algal.2014.11.011