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Biochemical composition of three algal species proposed as food for captive freshwater mussels

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Abstract

To identify potential diets for rearing captive freshwater mussels, the protein, carbohydrate (CHO), and lipid contents of two green algae, *Neochloris oleoabundans*, *Bracteacoccus grandis*, and one diatom, *Phaeodactylum tricornutum*, were compared at different growth stages. The fatty acid and sterol composition were also identified. Protein was greatest (55–70%) for all species at late log growth stage (LL), and declined in late stationary (LS) growth. CHO was greatest at LS stage for all species (33.9–56.4% dry wt). No significant change in lipid levels occurred with growth stage, but tended to increase in *N. oleoabundans*. Mean lipid content differed significantly in the order: *N. oleoabundans* > *P. tricornutum* > *B. grandis*. Total fatty acids (TFA) were higher at LS stage compared to other stages in the two green algae, and stationary stage in the diatom. Mean unsaturated fatty acids (UFA) as %TFA was significantly higher in *N. oleoabundans* than the other species. The green algae contained high percentages of C-18 polyunsaturated fatty acids (PUFAs), while the diatom was abundant in C-16 saturated and mono-unsaturated fatty acids and C-20 PUFA fatty acids. Growth stage had no effect on sterol concentration of any species. *B. grandis* showed significantly higher sterol levels than the other species except *P. tricornutum* at S stage. *B. grandis* was characterized by predominantly Δ^5, C-29 sterols, while *N. oleoabundans* synthesized $\Delta^{5,7}$, $\Delta^{5,7,22}$, and Δ^7, C-28 sterols. *P. tricornutum* produced primarily a $\Delta^{5,22}$, C-28 sterol, and a small amount of a $\Delta^{7,22}$, C-28 sterol.

Introduction

Microalgae play an important role in mariculture as food for many molluscs, crustaceans and some fish. Growth and development of these animals reared on microalgae are dependent on the proportion and availability of the biochemical constituents and digestibility of the cells (Chu and Dupuy 1981; Wikfors et al. 1992). The nutritional requirements of marine bivalves in particular, varies seasonally with changing reproductive condition and changes in the quality and quantity of food (Hawkins and Bayne 1985; Kreeger et al. 1995).

The biochemical composition of algae varies with species, light, temperature, and growth stage. Variation in biochemical composition due to growth stage is frequently related to culture age and nutrient depletion, particularly if an organism is grown in batch culture (Harrison et al. 1977; Morris et al. 1983). Typically, algal cultures become depleted in nutrients, as they enter stationary stages of growth, and total lipid and CHO increase while protein declines (Ogbonna

and Tanaka 1996; Zhu et al. 1997; Lourenco et al. 1997). Changes in lipid classes also have been observed as a function of growth stage. In general, phospholipids and glycolipids decline and triacylglycerol and free fatty acids increase (Lourenco et al. 1997; Zhu et al. 1997; Alonso et al. 2000). Fatty acids are structural components of many lipids, and the types and amounts of fatty acids vary considerably among algae. Sterols are also lipid components of eukaryotes, associated with functions such as hormone synthesis and hormone regulation (Nes 1974). Because aquatic consumers such as freshwater mussels have a defined suite of nutritional requirements, the relative balance of all these biochemical components is important in identifying quality algal diets for the culture and propagation of freshwater mussels.

The gross biochemical composition (protein, carbohydrate and lipids) of three species of freshwater algae (2 greens, 1 diatom) were characterized. These algae are being considered as diets for the captive care of endangered freshwater mussels, since they all appear digestible by them (Gatenby et al. 1997; Gatenby 2000). Because bivalves may require exogenous sources of PUFAs and sterols, the fatty acid and sterol composition of the lipid fraction of these algae at four different growth stages also was identified.

Materials and methods

Microalgal cultures

The green algae *Bracteacoccus grandis* (UTEX #1246) and *Neochloris oleoabundans* (UTEX #1185) and the diatom *Phaeodactylum tricornutum* (UTEX #640) were obtained from the University of Texas Culture Collection of Algae, Austin, TX, USA. *Neochloris oleoabundans* and *Phaeodactylum tricornutum* were cultured in Bold's Basal Medium with added soil extract (Nichols 1973). *Bracteacoccus grandis* was cultured in ″Our *Chlorella* Medium″ (OCM) (Behrens et al. 1989). All media were prepared fresh from respective dry chemicals. Starter cultures of 3 mL (in mid-log stage) were inoculated to 30 mL medium in 100 mL test tubes. The tubes were placed in a water bath maintained at 17 ± 1 °C under continuous cool white fluorescent light of 60 μmol m^{-2} s^{-1} photon flux. The cultures were aerated with approximately 1.5% CO_2 in air.

Algae were harvested at early log (EL), late log (LL), stationary (S), and late stationary (LS) growth stages. Growth rates were monitored by microscopy cell counts. Growth curves (not shown) were constructed to confirm growth stage identification. For example, *N. oleoabundans* reached LL stage by 10 d. Nine growth trials were conducted, containing 12 replicate cultures of each species. All ″replicate″ cultures were harvested at the same time within each growth trial by removing the entire volume of the culture under a fume hood. Replicate cultures for each species within each growth trial were pooled, and thus were transferred to acid washed glassware. Triplicate samples of the wet material were allocated to each assay (protein, carbohydrate, lipid, dry wt, and ash-free dry wt). In early log-phase, however, growth trials were repeated three times in order to obtain enough material for triplicate assays of each component. The samples of algae to be analyzed for protein and carbohydrate content were then freeze-dried.

Protein analysis

The protein content of 4 mg freeze-dried alga was determined spectrophotometrically using a Pierce test kit (BCA 23225) based on the procedure of Lowry et al. (1951) and Kreeger et al. (1997), and standardized with bovine serum albumin. Samples were prepared on microplates and analyzed at 562 nm with a microplate reader (Thermomax Molecular Devices Corporation, Sunnyvale, CA, USA).

Carbohydrate (CHO) analysis

The CHO content of 15 mg freeze-dried alga was determined spectrophotometrically using a modification of the procedure by Pakulski and Benner (1992) and standardized with potato starch (soluble grade, Baker, 4006-4) (Huang et al. 2002). After color development, a 200 μL sample of the supernatant was transferred to three replicate wells in two 96-well microplates and absorbance read immediately at 665 nm with a microplate reader (Thermomax Molecular Devices Corporation, Sunnyvale, CA, USA).

Lipid analysis

Lipids were extracted from 500–1000 mg wet wt of alga, processed and dried under liquid N_2 using the method of Yan et al. (1997). The lipids were separated into specific fractions using silica gel G thin layer

Table 1. Biochemical assay sample sizes per species per growth phase. EL = early log phase growth, LL = late log, S = stationary, LS = late stationary.

	Growth Stage	Protein	Carbohydrate	Lipid	Ash
Bracteacoccus grandis	EL	8	7	9	6
	LL	3	2	4	6
	S	2	3	3	6
	LS	2	4	4	6
Neochloris oleoabundans	EL	3	2	3	6
	LL	4	7	3	6
	S	3	3	9	6
	LS	4	4	4	6
Phaeodactylum tricornutum	EL	2	2	3	6
	LL	3	4	5	6
	S	1		3	6
	LS	1	2	1	6

chromatography (TLC). Fatty acid and total sterol were determined by co-chromatography using a mixture containing authentic lipid standards (Supelco, Bellefonte, PA, USA). Volatile methyl esters and trimethysilylether derivatives were prepared for total fatty acids and sterols, respectively, following removal from TLC plates and analyzed by gas liquid chromatography (GLC), GLC/mass spectrometry, as previously described. Identification and quantification of fatty acids and sterols was based on GLC retention times compared to known standards and published literature (Patterson 1971). Quantification of fatty acids and sterols was determined using internal standards of heptadecanoic acid (C:17) and coprostanol, respectively.

Electron ionization mass spectra were obtained for the fatty acids and sterols extracted from samples of each species. A Hewlett-Packard 5790 gas chromatograph fitted with a splitless injector and a 30 m × 0.32 mm (i.d.) HP-5 capillary column coated with a 5% diphenyldimethylsiloxane phase was used for sterol analysis. A 30 m × 0.32 mm (i.d.) Omegawax capillary column (Supelco, Bellefonte, PA, USA) was used to analyze the fatty acids. Both columns were held at 95 °C for 1 min, then temperature-programmed at a rate of 15 °C min^{-1} to 300 °C. Helium was used as the carrier gas at a pressure of 124 kPa for fatty acids and 103 kPa for sterols. Electron ionization spectra were obtained at 70 eV with a source block temperature of 200 °C using a VG-7070E-HF mass spectrometer (Micromass, Manchester, UK).

Statistical analysis

Nine growth trials were conducted, containing 12 replicates for each species. Material harvested from replicate cultures within each growth trial were pooled. Pooled material was subdivided, allowing for at least one sample from each growth trial per biochemical assay per species. Although growth trials were repeated to increase the number of samples analyzed for each species at each stage of growth, differences in weight among algae and occasional contaminated cultures resulted in unequal sample sizes between species. In addition, stationary phase samples for *P. tricornutum* intended for CHO analysis were improperly microplated, yielding invalid data. Sample sizes for each assay per species per growth phase are presented in Table 1. Four replicate spectrophotometric measurements of the CHO and protein data were made. A test of equal variance was conducted for each analysis (tests described below).

Biochemical composition was calculated as a percentage of total algal dry weight from the absolute concentrations for each biochemical component. The raw percentage data for protein, CHO, lipid, and ash was then normalized to 100% of total dry wt. The weight of various nucleic acids and other organic components not accounted for in the protein, CHO, and lipid analyses was assumed negligible. Normalized percentage data were arcsine square root-transformed prior to all statistical analyses (Sokal and Rohlf 1981). Absolute concentrations (μg) of fatty acids and sterols also were calculated as a percent of total lipid wt and arcsin square root-transformed prior

to statistical analyses. Concentrations (μg mg^{-1} lipid) of saturated and unsaturated fatty acids (also, arc-sin transformed) were compared among species, among growth stages, and within species among growth stages. Concentration values and percent composition values were analyzed using two-way analysis of variance (ANOVA), with growth stage and species as the dependent variables. When no growth stage effect was detected by the two-way ANOVA, one-way ANOVA followed by Fisher's Protected Least Significant Difference (FPLSD) compared differences among species (Lentner 1993). The ANOVA F-test is very robust to mild departures from homogeneous variances, especially when the data have been transformed to correct for variability arising from unequal sample sizes (Lentner 1993). Kruskal-Wallis test for homgeneous variance, however, was conducted in combination with the one-way ANOVA's. One-way ANOVA and FPLSD also were conducted to compare differences among growth stages within a species.

Results

Protein

Overall, there was no effect of species or growth stage on protein content (% of algal dry wt) in the algae (two-way ANOVA, $p = 0.488$). However, protein declined at LS for *Bracteococcus grandis* and *Neochloris oleoabundans* (one-way ANOVA and FPLSD, $p < 0.05$) compared to earlier stages (Figure 1). Although the amount of protein in *Phaeodactylum tricornutum* appeared least in the LS, the value did differ significantly from the other growth stages (FPSLD, $p = 0.52$). Interspecies comparisons showed that the protein content of *B. grandis* was greater than the other species at S stage, and *N. oleoabundans* was significantly lower than *P. tricornutum* at LS, but not lower than *B. grandis*. Ignoring growth phase, no difference occurred in the overall mean protein content among species (one-way ANOVA, $p = 0.308$). The protein content of growth stages ranged from 45.2 ± 19.3 to 54.1 ± 17.9% of algal dry wt. (Figure 2).

Carbohydrate

There was a significant effect of growth stage and species on the carbohydrate (CHO) content (% dry wt) (two-way ANOVA). The CHO content increased at LS in all three species, compared to earlier growth

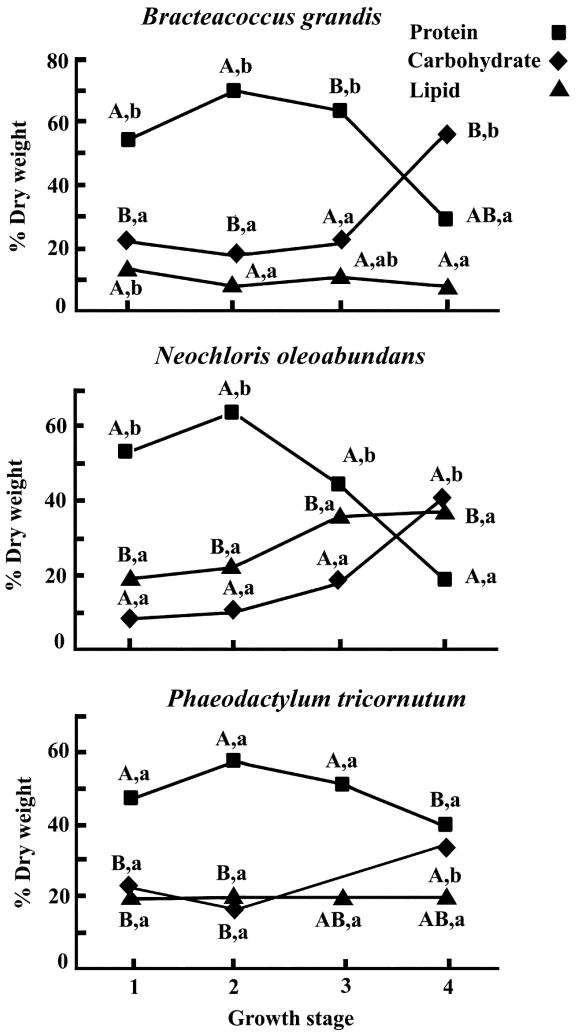

Figure 1. Carbohydrate, protein and lipid contents of *Bracteacoccus grandis, Neochloris oleoabundans* and *Phaeodactylum tricornutum* at different growth stages (1 = early log/log; 2 = late log; 3 = stationary; 4 = late stationary). Values within each species and chemical category followed by the same lower case letter are not significatly different ($p \leqslant 0.05$). Values across species for the same chemical category and growth stage followed by the same upper case letter are not significant ($p \leqslant 0.05$).

stages. The CHO content also differed among species within a growth stage. The CHO contents of *B. grandis* and *P. tricornutum* at EL and LL were greater than in *N. oleoabundans*. At LS, however, the CHO content in *B. grandis* (56.4%) was greater than in *P. tricornutum* and *N. oleoabundans*, while the latter two were similar (Figure 1). Indeed, the overall mean CHO content in *B. grandis* was higher than in the other species (Figure 2). CHO levels ranged from

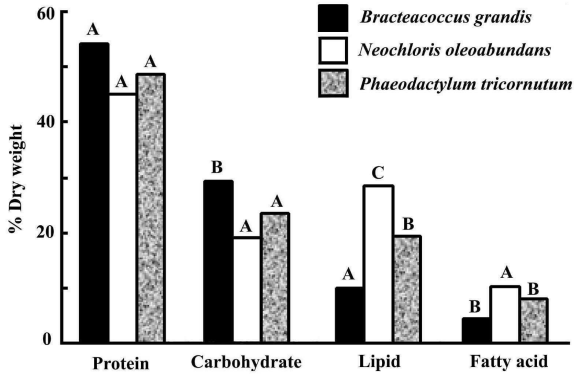

Figure 2. Mean percentages of carbohydrate, protein, lipid, and fatty acid across all growth stages of *Bracteacoccus grandis, Neochloris oleoabundans* and *Phaeodactylum tricornutum.* Values across species within a chemical category followed by the same upper case letter are not significant ($p \leqslant 0.05$).

8–56% dry wt depending on growth stage and species.

Lipids

The lipid content (% dry wt) differed between species (p = 0.0), but was apparently not influenced by the growth phase (two-way ANOVA, p = 0.314), (Figure 1). In *N. oleoabundans*, however, the lipid content increased from 18.9% of algal dry wt at EL to 37.2% of algal dry wt at LS stage (one-way ANOVA, p = 0.093). In *B. grandis*, there was a decreasing trend in lipid content with growth stage (p = 0.016). *N. oleoabundans* produced more lipid throughout the study than *B. grandis*, although the values were not significantly higher than those for *P. tricornutum* (Figure 1). *B. grandis* contained the least amount of lipid (10.0 ± 2.5%) compared to *P. tricornutum* (19.4 ± 4.6) and *N. oleobundans* (28.5 ± 9.4), and all were significantly different (Figure 2).

Overall, there was no effect of growth stage or species on total fatty acids (TFA) (two-way ANOVA, p = 0.920). Some differences in TFA among species were apparent at S and LS stages (one-way ANOVA, FPLSD). TFA concentration was greatest in *P. tricornutum* (632.2 ± 120.3 μg mg lipid^{-1}) at S stage (p = 0.018), compared to the green algae at the same growth stage (Figure 3). TFA concentration was higher at LS in both green algae compared to *P. tricornutum* at LS. Interestingly, the TFA concentration was lowest in *N. oleoabundans* at S stage but was highest at LS (540.2 ± 152.2 μg· mg lipid^{-1}) (p = 0.0189). No significant differences were observed in

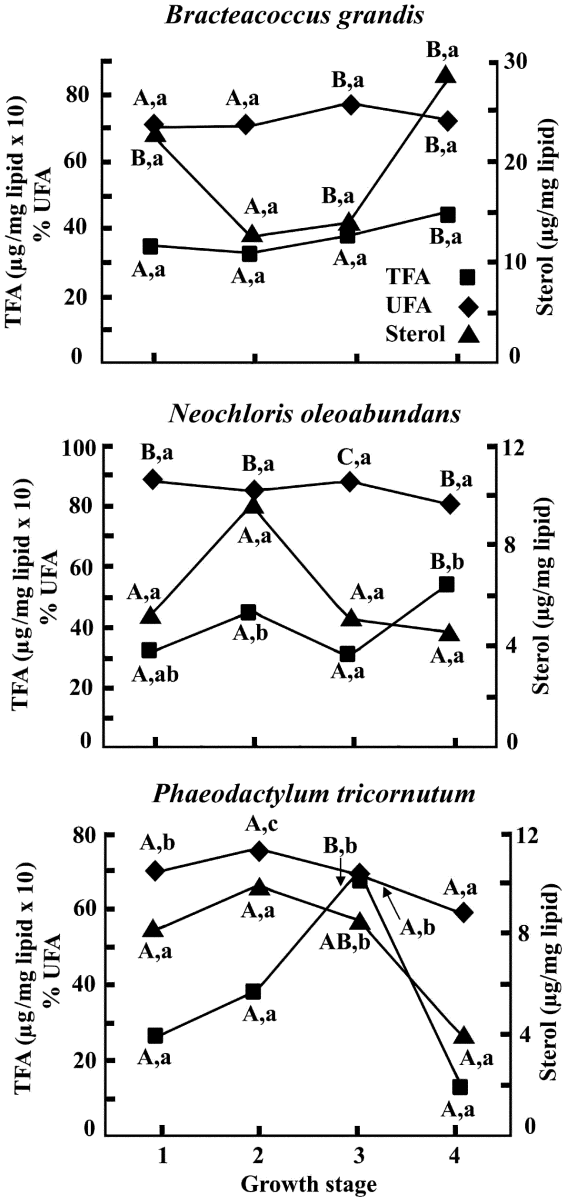

Figure 3. Total fatty acid, unsaturated fatty acid and sterol content of *Bracteacoccus grandis, Neochloris oleoabundans* and *Phaeodactylum tricornutum* at different growth stages (1 = early log/log; 2 = late log; 3 = stationary; 4 = late stationary). Values within each species and chemical category followed by the same lower case letter are not significant ($p \leqslant 0.05$). Values across species for the same chemical category and growth stage followed by the same upper case letter are not significant ($p \leqslant 0.05$).

the overall mean TFA (μg· mg lipid^{-1}) concentration for all growth stages among species. However, when expressed as a percentage of dry wt, *B. grandis* and *P. tricornutum* exhibited lower amounts of TFA than *N. oleoabundans* (Figure 2).

Neochloris oleoabundans contained the greatest amount of unsaturated fatty acids (UFA) as % TFA over the other two algae, with the exception that UFA were not different from that of *B. grandis* at LS stage (Figure 3). In addition, no significant differences were observed between growth stages within the species of green algae. *P. tricornutum*, however, had higher UFA in the LL stage compared with other growth stages, and were lowest in the LS stage. Overall, the total mean UFA concentration (%TFA) of *N. oleoabundans* (85.0 ± 6.2) was greater than that of either *B. grandis* (72.5 ± 9.5) or *P. tricornutum* (68.9 ± 6.3). In contrast, no differences in saturated fatty acids (SAFA) were observed between growth stages within the species of green algae, but *P. tricornutum* had significantly higher SAFA at LS compared to other stages. In addition, the overall mean SAFA concentration (%TFA) of *N. oleoabundans* (14.6 ± 6.2) was significantly lower than that of either *B. grandis* (27.5 ± 9.5) or *P. tricornutum* (31.9 ± 6.3) (Data not shown).

The most abundant fatty acids in *B. grandis* were 16:0, 18:1, 18:2, and 18:3 ($\geqslant$ 20%), with smaller amounts of 16:1, 16:2, and 18:0 ($\leqslant$ 5%). The most abundant fatty acids in *N. oleoabundans* were 16:1, 18:2, and 18:3 ($\geqslant$ 15%). Smaller amounts of 14:0, 15:0, 16:0, 16:2, 16:3, 20:1, and 24:0 also were detected ($\leqslant$ 7%). The most abundant fatty acids in *P. tricornutum* were 16:0, 16:1, and 20:5 ($\geqslant$ 19%). Small amounts of 14:0, 16:2, 18:0, 18:1, 18:2, 18:3, 18:4, 20:4, and 24:0 also were observed ($\leqslant$ 5%). The green algae characteristically exhibited higher percentages of polyunsaturated 18-C fatty acids; whereas, *P. tricornutum* exhibited high percentages of saturated and mono-unsaturated C-16 fatty acids. Also unique to *P. tricornutum* was the presence of high percentages of polyunsaturated 20-C fatty acids (Figure 4).

There was an effect of species (two-way ANOVA, $p = 0.001$) but no effect of growth stage ($p = 0.741$) on the sterol content in the algae. The sterol concentration of *B. grandis* was higher for all stages of growth compared to the other algal species. However, these differences were only significant at the EL and LS stages (one-way ANOVA). One exception was that *B. grandis* was higher at the S stage compared to *N. oleoabundans*. The total mean sterol concentration of *P. tricornutum* (7.6 ± 3.6 μg mg^{-1} lipid) and *N. oleoabundans* (6.4 ± 5.5 μg mg^{-1} lipid) was similar, and significantly less than *B. grandis* (19.6 ± 12.6 μg mg^{-1} lipid) ($p = 0.0$).

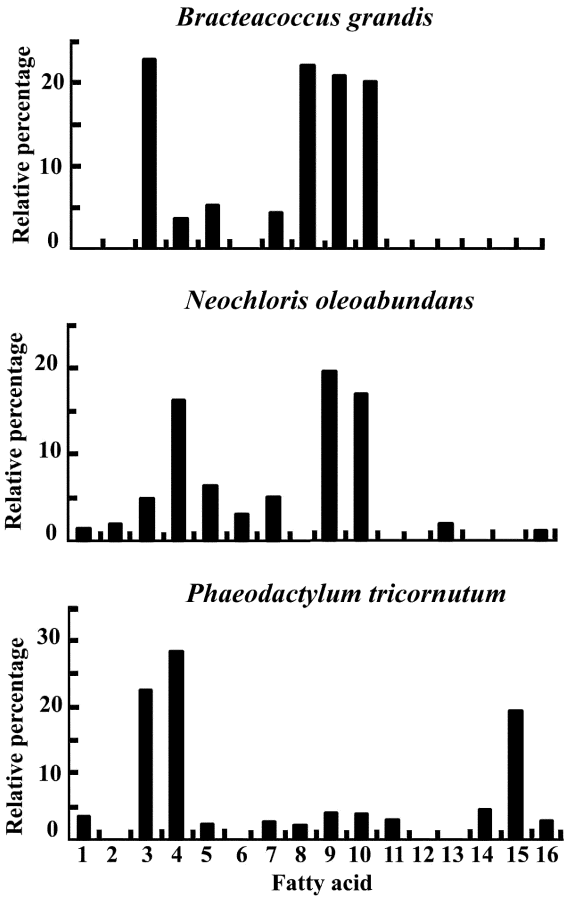

Figure 4. Relative percentages of fatty acids in *Bracteacoccus grandis, Neochloris oleoabundans* and *Phaeodactylum tricornutum*. (1 = 14:0, 2 = 15:0, 3 = 16:0, 4 = 16:1, 5 = 16:2, 6 = 16:3, 17 = 18:0, 8 = 18:1, 9 = 18:2, 10 = 18:3, 11 = 18:4, 12 = 20:0, 13 = 20:1, 14 = 20:4, 15 = 20:5, 16 = 24:0).

Three major sterols were identified in each of *N. oleoabundans* and *P. tricornutum* and six in *B. grandis* (Figure 5). *B. grandis* contained predominantly $\Delta^{5,22}$-stigmastenol, Δ^{5}-stigmastenol, and Δ^{5}-ergostenol, with lesser amounts (< 5% total sterol content) of Δ^{7}-ergostenol, $\Delta^{8,9}$-stigmastenol, and Δ^{7}-chondrillasterol. *N. oleoabundans* contained abundant $\Delta^{5,7,22}$-ergostatrienol (45.3% total sterol content), followed by similar amounts of $\Delta^{5,7}$-ergostadienol and Δ^{7}-ergostenol (28.1% and 26.6%, respectively). The predominant sterol in *P. tricornutum* was $\Delta^{5,22}$-ergostadienol (brassicasterol, 89.5%) with $\Delta^{7,22}$-ergostadienol present in lower percentages (10.5%). Trace amounts of Δ^{5}-cholestenol (cholesterol) also were detected in *P. tricornutum*.

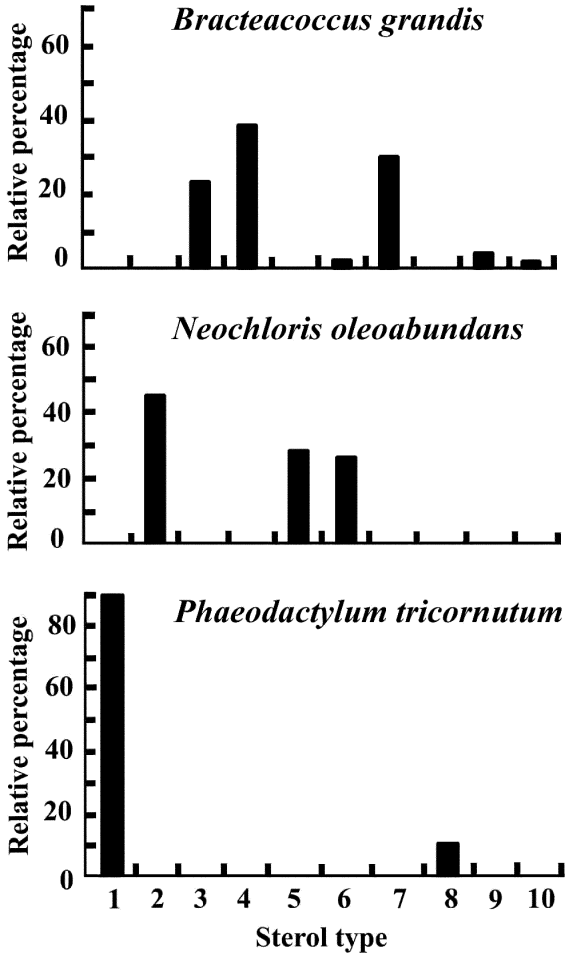

Figure 5. Types and relative percentages of sterols in *Bracteacoccus grandis, Neochloris oleoabundans* and *Phaeodactylum tricornutum* (1 = $\Delta^{5,22}$-ergostadienol; 2 = $\Delta^{5,7,22}$-ergostatrienol; 3 = Δ^{5}-ergostenol; 4 = $\Delta^{5,22}$-stigmastenol; 5 = $\Delta^{5,7}$-ergostadienol; 6 = Δ^{7}-ergostenol; 7 = Δ^{5}-stigmastenol; 8 = $\Delta^{7,22}$-ergostadienol; 9 = $\Delta^{8,9}$-stigmastenol; 10 = Δ^{7}-chondrillasterol).

Discussion

The algae tested were selected on the basis of their potential suitability as food for cultured freshwater mussels. Their cell size, good growth under a variety of environmental conditions in freshwater media (Tornabene et al. 1983; Yongmanitchai and Ward 1991; Arredondo-Vega et al. 1995), and previous testing as food (Gatenby et al. 1997; Gatenby 2000) were considered. Generally, as algae enter the stationary stages of growth, total lipid and CHO increase and protein declines (Ogbonna and Tanaka 1996; Zhu et al. 1997; Lourenco et al. 1997). Lipid content, however, appears to be the most variable among these components, is highly responsive to sources and concentrations of nitrogen, and varies with species. Similar findings were obtained in this study, i.e. increase in CHO, variability in lipid content, and decrease in protein with advancing growth stage. The prevalence of these biochemical constituents in the three algae are next discussed in relation to the known or suspected dietary needs of bivalve molluscs.

Protein

For animal consumers that feed on microalgae, the dietary demand for gross biochemical constituents will likely vary among species and life stages (e.g. bivalve molluscs see Hawkins and Bayne (1985) and Kreeger et al. (1995)). Proteins are the fundamental building blocks for tissue biosynthesis and enzyme production in all animals. Thus, dietary protein must meet these demands for tissue production and metabolic processes. Protein is generally considered most important to the rapidly growing juvenile life stage, followed by adults undergoing gametogenesis (Langton et al. 1977; Wikfors et al. 1992; Kreeger 1993), but protein demand appears to vary interspecifically among different consumers. For example, Wikfors et al. (1984) found that protein levels ranging from 15.6% to 57.4% of algal dry wt were not limiting to growth of juvenile oysters (*C. virginica*). Kreeger and Langdon (1993), however, reported that growth of juvenile mussels (*Mytilus trossulus*) was constrained when dietary protein contents dropped below 40%.

Protein is typically the major biochemical component of algae (Brown and Jeffrey 1992; Wikfors et al. 1992) although as already mentioned, growth medium and growth stage will affect biochemical composition (Leonardos and Lucas 2000). Rapidly growing cells are characterized by a high protein and low CHO content, and when cells have reached stationary phase, more carbon is incorporated into CHO and/or lipids (Piorreck and Pohl 1984; Henderson and Sargent 1989; Zhu et al. 1997). Protein levels that ranged from about 19% to nearly 70% of cellular dry wt depending on growth stage were observed in this study. Based on reports for marine bivalves, it would appear that the protein content of the 3 species of algae examined here would be sufficient for freshwater mussels.

Carbohydrate

Carbohydrates are presumed the primary energy reserve in adult bivalves; whereas, the larvae accumulate lipids in the form of triacylglycerols as an energy source for developing organelles and metamorphosis (Flaak and Epifanio 1978; Wikfors et al. 1984; Enright et al. 1986). Both juvenile and adult oysters use CHO as a primary energy source, with glycogen representing 40–50% of their total CHO content (Holland and Spencer 1973; Wikfors et al. 1984). Castell and Trider (1974) varied the CHO and protein content of oyster diets and observed a higher glycogen production in adult eastern oysters (*Crassostrea virginica*) fed 60% CHO than diets with 20% CHO. A high CHO diet was of little use, however, to juvenile oysters (*Ostrea edulis*) when protein became limiting (Enright et al. 1986). Similarly, Wikfors et al. (1992) found no strong correlation between CHO in the diet and the growth rate of juvenile clams; rather, protein and lipid had a stronger effect on growth rate. An apparent biochemical basis exists for the nutritional value of certain algal species to different species of bivalves and at different life stages. Unfortunately, the nutritional and energy requirements of freshwater mussels are unknown.

Brown and Jeffrey (1992) reported CHO levels ranging from 5.9%–16.7% in 10 species of Chlorophyceae and Prasinophyceae. Wikfors et al. (1992) reported CHO values from 6.5%–31% of algal dry wt for 19 unialgal bivalve diets from the Prasinophyceae and diatoms. Changes in CHO content have been attributed to differences in growth medium, especially nutrient levels, with N-deficient media producing algae with the highest CHO (Brown and Jeffrey 1992). The CHO levels of the species studied by us were similar, and also increased with growth phase, concomitant with a probable decline in nutrients during growth in batch culture. Their CHO contents were in line with other bivalve algal diets. It would appear, therefore, that these species would be suitable for meeting the energy maintenance requirements of freshwater mussels.

Lipids

Finally, lipids are considered very important during gametogenesis for gonad maturation and especially in females to provide an energy source for subsequent embryo development (Pollero et al. 1983). Although aquatic invertebrates synthesize saturated and monoenoic fatty acids, it has not been shown conclusively whether they can synthesize *de novo* polyunsaturated fatty acids (PUFAs) which are considered essential for growth, development, and cellular function (Morris and Sargent 1973; Holland 1978; Chu and Greaves 1991). In addition, many marine bivalves show a low capacity for desaturating fatty acids to PUFAs; thus, they satisfy most of their essential fatty acid requirements through dietary sources (Kanazawa et al. 1979; Chu and Greaves 1991; Zhukova et al. 1998). Little is known of the lipid biosynthesis capabilities of freshwater molluscs, and even less on their quantitative and qualitative lipid requirements. We assume that like other aquatic organisms, they will require dietary lipids (unsaturated and saturated fatty acids and sterols) to provide precursors for chain elongation of essential highly unsaturated fatty acids, synthesis of hormones, and as an energy source in developing embryos and larvae (Kanazawa et al. 1979; Waldock and Holland 1984; Cohen et al. 1988; Napolitano et al. 1993). Cholesterol is viewed as the most important sterol in aquatic molluscs because it is required for the synthesis of membranes and for use in the production of other steroids necessary for reproductive development. For the most part, bivalve molluscs have limited abilities to synthesize sterols (Popov et al. 1981; Teshima and Patterson 1981; Holden and Patterson 1991). Sterols are presumed, therefore, to be an essential dietary requirement obtained from plant sources such as algae (Berenberg and Patterson 1981; Tsitsa-Tzardis et al. 1993; Patterson et al. 1994). Knowing the sterol composition of algae proposed as diets for the culture of freshwater mussels or other cultured organisms becomes important relative to understanding their nutrition and successfully rearing them in captivity.

In our study, the three species showed very different sterol compositions. *Phaeodactylum tricornutum* had predominantly a C-28 sterol, brassicasterol, which has been reported previously in this species (Rubinstein and Goad 1974a; Orcutt and Patterson 1975). *Neochloris oleoabundans* possessed three primary sterols, including $\Delta^{5,7,22}$ -ergostatrienol, $\Delta^{5,7}$ -ergostadienol, and Δ^{7} -ergostenol. Tornabene et al. (1983) detected seven different sterols in *N. oleoabundans*, only four of which were in sufficient quantity to tentatively identify. The most abundant sterol was identified as Δ^{7} -ergostenol. A second sterol was probably a C-28 sterol with a double bond in rings B and D. Specific positions of double bonds were not indicated. A third sterol was thought to have three

double bonds in the ring system, but insufficient material precluded a good mass spectrum. A fourth sterol was tentatively identified as cholest-14-en-3β-ol. Except for the latter sterol, it is quite possible the sterols identified by us in *N. oleoabundans* are the same as those tentatively identified by Tornabene et al. (1983).

Wright et al. (1980) found Δ^7-ergostenol, $\Delta^{7,22}$ chondrillastenol, and Δ^7-chondrillastenol in *Bracteococcus cinnabarinus*; the latter, which is a 29 carbon sterol, comprised 70–80% total sterols. Three major sterols (Δ^5-ergostenol, $\Delta^{5,22}$-stigmastenol, and Δ^5-stigmastenol) and three minor sterols (Δ^7-ergostenol, $\Delta^{8,9}$-stigmastenol, and Δ^7-chondrillastenol) were identified by us in *B. grandis*; over 90% of its sterol consisting of Δ^5, C-28 (23%) and C-29 (67%) sterols. About equal amounts of Δ^7, C-28 (5%) and C-29 (5%) sterols were detected. This study is the first report of sterols in *B. grandis*. Considering the sterol diversity in these two species of *Bracteacoccus*, it would be of value to determine the sterol composition of other congeneric species. Indeed, the sterol composition of the *Chlorella* species has proven quite diverse and useful as a chemotaxonomic tool (Holden and Patterson 1982).

Changes in lipid content and lipid classes have been observed as a function of algal growth stage. Overall, TFA increase with advancing growth stage, while the degree of unsaturation declines and the degree of saturation increases (Cohen et al. 1988; Zhu et al. 1997; Alonso et al. 2000). In our study TFA tended to increase as the growth stage advanced, but the effect was only significant in *P. tricornutum*. However, TFA declined in the LS stage in this species. Also, UFA and SAFA did not change in either of the green algae, but UFA declined and SAFA increased significantly in *P. tricornutum*, with advancing growth stage. The lack of highly unsaturated fatty acids in the green algae was not unexpected. Although we did observe small amounts of 20:1 in *N. oleoabundans*, this and *B. grandis* produced mostly C-18 UFA, as did *P. tricornutum*. Unlike the green algae, the diatom contained a significant amount of the highly unsaturated fatty acid, eicosapentaenoic acid (20:5).

Freshwater mussels undergo significant developmental changes from their parasitic larval stage to a free-living, pedal-feeding juvenile to a filter-feeding adult. As more is learned about their elemental and biochemical requirements at different stages, it will become possible to target these nutritional demands with particular algal species harvested at the appropriate growth phase. It is believed that a more optimal balance of macro- and micro-nutrients is provided by a mixed than a unialgal diet (Enright et al. 1986; Wikfors et al. 1992). Although, the three algae examined have received only limited testing as foods for freshwater mussels (Gatenby et al. 1997; Gatenby 2000), their protein, CHO, TFA, PUFA and sterol contents during all growth stages suggest they will be suitable as dietary species. Efforts to conserve and propagate endangered freshwater mussels would benefit from research designed to determine the digestibility of these algae by the mussels, and to define the specific nutritional demands of mussels for key biochemical compounds such as protein, total lipid, sterols and fatty acids.

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References

Alonso D.L., Belarbi E.-H., Fernández-Sevilla J.M., Rodrigues-Ruiz J. and Grima E.M. 2000. Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga *Phaeodactylum tricornutum*. Phytochemistry 54: 461–471.

Arredondo-Vega B.O., Band-Smith C.J. and Vazquez-Duhalt R. 1995. Biochemical composition of *Neochloris oleoabundans* adapted to marine medium. Cytobios 83: 201–205.

Behrens P.W., Bingham S.E., Hoeksema S.D., Cohoon D.L. and Cox J.C. 1989. Studies on the incorporation of CO_2 into starch by *Chlorella vulgaris*. J. Appl. Phycol. 1: 123–130.

Berenberg C.J. and Patterson G.W. 1981. Relationship between dietary phytosterols and the sterols of wild and cultivated oyster (*Crassostrea virginica*). Lipids 16: 276–278.

Brown M.R. and Jeffrey S.W. 1992. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars, and pigments. J. Exp. Mar. Biol. Ecol. 161: 91–113.

Castell J.D. and Trider D.J. 1974. Preliminary feeding trials using artificial diets to study the nutritional requirements of oysters (*Crassostrea virginica*). J. Fish. Res. Bd Canada 31: 95–99.

Chu F.L.E. and Dupuy J.L. 1981. The fatty acid composition of three unicellular algal species used as food sources for larvae of the American oyster (*Crassostrea virginica*). Lipids 15: 356–364.

Chu F.L.E. and Greaves J. 1991. Metabolism of palmitic, linoleic, and linolenic acids in adult oyster, *Crassostrea virginica*. Mar. Biol. 110: 229–236.

Cohen Z., Vonshak A., Boussiba S. and Richmond A. 1988. Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation to growth rate. J. Phycol. 24: 328–332.

Enright C.T., Newkirk G.F., Craigie J.S. and Castell J.D. 1986. Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. J. Exp. Mar. Biol. Ecol. 96: 1–13.

Flaak A.R. and Epifanio C.E. 1978. Dietary protein levels and growth of the oyster *Crassostrea virginica* Gmelin. Aquaculture 11: 13–29.

Gatenby C.M. 2000. A study of holding conditions, feed ration, and algal foods for the captive care of freshwater mussels. PhD Dissertation, Virginia Polytechnic Institute & State University, Blacksburg, USA.

Gatenby C.M., Parker B.C. and Neves R.J. 1997. Growth and survival of juvenile rainbow mussels, *Villosa iris* (Bivalvia: Unionidae), reared on algal diets and sediment. Am. Malacol. Bull. 14: 57–66.

Harrison P.J., Conway H.L., Holmes R.W. and Davis C.O. 1977. Marine diatoms grown in chemostats under silicate or ammonium limitation. III. Cellular chemical composition and morphology of *Chaetoceros debilis*, *Skeletonema costatum* and *Thalassiosira gravida*. Mar. Biol. 43: 19–31.

Hawkins A.J.S. and Bayne B.L. 1985. Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis* budgets, conversion efficiencies and maintenance requirements. Mar. Ecol. Progr. Ser. 25: 181–188.

Henderson R.J. and Sargent J.R. 1989. Lipid composition and biosynthesis in ageing cultures of the marine cryptomonad, *Chroomonas salina*. Phytochemistry 28: 1355–1361.

Holden M.J. and Patterson G.W. 1982. Taxonomic implication of sterol composition in the genus *Chlorella*. Lipids 17: 215–219.

Holden M.J. and Patterson G.W. 1991. Absence of sterol biosynthesis in oyster tissue culture. Lipids 26: 81–82.

Holland D.L. 1978. Lipid reserves and energy metabolism in the larvae of benthic marine invertebrates. In: Malins D.C. and Sargent J.R. (eds), Biochemical and Biophysical Perspectives in Marine Biology, 4. Academic Press, New York, pp. 85–123.

Holland D.L. and Spencer J.S. 1973. Biochemical changes in fed and starved oysters, *Ostrea edulis* L. during larval development, metamorphosis and early spat growth. J. Mar. Biol. Assoc. UK 53: 287–298.

Huang S.C., Newell R.I.E. and Kreeger D.A. 2002. Seston as a food resource for the ribbed mussel (*Geukensia demissa*) in a North American, mid-Atlantic salt marsh. Estuar. Coast. Mar. Sci. (in press).

Kanazawa A.S., Teshima S. and Ono K. 1979. Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. Comp. Biochem. Physiol. 63: 295–298.

Kreeger D.A. 1993. Seasonal patterns in utilization of dietary protein by the mussel *Mytilus trossulus*. Mar. Ecol. Progr. Ser. 95: 215–232.

Kreeger D.A., Goulden C.E., Kilham S.S., Lynn S.G., Datta S. and Interlandi S.J. 1997. Seasonal changes in the biochemistry of lake seston. Freshwat. Biol. 38: 539–554.

Kreeger D.A., Hawkins A.J.S., Bayne B.L. and Lowe D.M. 1995. Seasonal variation in the relative utilization of dietary protein for energy and biosynthesis by the mussel *Mytilus edulis*. Mar. Ecol. Progr. Ser. 126: 177–184.

Kreeger D.A. and Langdon C.J. 1993. Effect of dietary protein content on growth of juvenile mussels, *Mytilus trossulus* (Gould1850). Biol. Bull. 185: 123–129.

Langton R.W., Winter J.E. and Roels O.A. 1977. The effect of ration size on growth and growth efficiency on the bivalve mollusc, *Tapes japonica*. Aquaculture 12: 283–292.

Lentner M. 1993. Experimental Design and Analysis. Valley Book Company, Blacksburg, VA, USA, 585 pp.

Leonardos N. and Lucas I.A.N. 2000. The nutritional value of algae grown under different culture conditions for *Mytilus edulis* L. larvae. Aquaculture 182: 301–315.

Lourenco S.O., Marquez U.M.L., Mancini-Filho J., Barbarino E. and Aidar E. 1997. Changes in biochemical profile of *Tetraselmis gracilis* I. Comparison of two culture media. Aquaculture 148: 153–168.

Lowry O.H., Rosenbrough N.H., Farr A.L. and Randall R.J. 1951. Protein measurements with folinphenol reagent. J. Biol. Chem. 193: 265–275.

Morris R.J. and Sargent J.R. 1973. Studies on the lipid metabolism of some oceanic crustaceans. Mar. Biol. 22: 77–83.

Morris R.J., McCartney M.J. and Robinson G.A. 1983. Studies of a spring phytoplankton bloom in an enclosed experimental ecosystem. I. Biochemical changes in relation to the nutrient chemistry of water. J. Exp. Mar. Biol. Ecol. 70: 249–262.

Napolitano G.E., Ackman R.G. and Silva-Serra M.A. 1993. Incorporation of sterols by the sea scallop *Placopecten magellanicus* (Gmelin) fed on microalgae. Mar. Biol. 117: 647–654.

Nes W.R. 1974. Role of sterols in membranes. Lipids 9: 596–611.

Nichols H.W. 1973. Growth media – freshwater. In: Stein J.R. (ed.), Handbook of Phycological Methods. Cambridge University Press, New York, pp. 13–14.

Ogbonna J.C. and Tanaka H. 1996. Night biomass loss and changes in biochemical composition of cells during light/dark cyclic culture of *Chlorella pyrenoidosa*. J. Ferm. Bioeng. 82: 558–564.

Orcutt D.M. and Patterson G.W. 1975. Sterol, fatty acid, and elemental composition of diatoms grown in chemically-defined media. Comp. Biochem. Physiol. 50: 579–583.

Pakulski J.D. and Benner R. 1992. An improved method for the hydrolysis and MBTHanalysis of dissolved and particulate carbohydrates in seawater. Mar. Chem. 40: 143–160.

Patterson G.W. 1971. Relation between structure and retention time of sterols in gas chromatography. Analyt. Chem. 43: 1165–1170.

Patterson G.W., Tsitsa-Tzardis E., Wikfors G.H., Ghosh P., Smith B.C. and Gladu P.K. 1994. Sterols of eustigmatophytes. Lipids 29: 661–664.

Piorreck M. and Pohl P. 1984. Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase. Phytochemistry 23: 217–223.

Pollero R.J., Irazu C.E. and Brenner R.R. 1983. Effect of sexual stages on lipids and fatty acids of *Diplodon delodontus*. Comp. Biochem. Physiol. 76: 927–931.

Popov S., Stoilov I., Marekov N., Kovachev G. and Andreev St. 1981. Sterols and their biosynthesis in some freshwater bivalves. Lipids 16: 663–669.

Rubinstein I. and Goad L.J. 1974a. Sterols of the siphonous marine alga, *Codium fragile*. Phytochemistry 13: 481–484.

Sokal R.R. and Rohlf F.J. 1981. Biometry. W. H. Freeman and Co., San Francisco, CA, USA, 859 pp.

Teshima S.-I. and Patterson G.W. 1981. Sterol biosynthesis in oyster, *Crassostrea virginica*. Lipids 16: 234–239.

Tornabene T.G., Holzer G., Lien S. and Burris N. 1983. Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. Enzyme Microb. Technol. 5: 435–440.

Tsitsa-Tzardis E., Patterson G.W., Wikfors G.H., Gladu P.K. and Harrison D. 1993. Sterols of *Chaetoceros* and *Skeletonema*. Lipids 28: 465–467.

Waldock M.J. and Holland D.L. 1984. Fatty acid metabolism in young oysters, *Crassostrea gigas*: polyunsaturated fatty acids. Lipids 19: 332–336.

Wikfors G.H., Ferris G.E. and Smith B.C. 1992. The relationship between gross biochemical composition of cultured algal foods and growth of the hard clam*, Mercenaria mercenaria* (L.). Aquaculture 108: 135–154.

Wikfors G.H., Twarog J.W. Jr and Ukeles R. 1984. The influence of chemical composition ofalgal food sources on growth of juvenile oysters, *Crassostrea virginica*. Biol. Bull. 167: 251–263.

Wright D.C., Berg L.R. and Patterson G.W. 1980. Effect of cultural conditions on the sterols and fatty acids of green algae. Phytochemistry 19: 783–785.

Yan J., Schmidt R.E. and Orcutt D.M. 1997. Influence of fortified seaweed extract and drought stress on cell membrane lipids and sterols of ryegrass leaves. Internat. Turfgrass Soc. Res. J. 8: 1356–1362.

Yongmanitchai W. and Ward O.P. 1991. Growth of and omega-3 fatty acid production by*Phaeodactylum tricornutum* under different culture conditions. Appl. Envir. Microbiol. 57: 419–425.

Zhu C.J., Lee Y.K. and Chao T.M. 1997. Effects of temperature and growth phase on lipid and biochemical composition of *Isochrysis galbana* TK1. J. Appl. Phycol. 9: 451–457.

Zhukova N.V., Imbs A.B. and Yi L.F. 1998. Diet-induced changes in lipid and fatty acid composition of *Artemia salina*. Comp. Biochem. Physiol. 120B: 499–506.