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Changes in the lipid composition and maximisation of the polyunsaturated fatty acid content of three microalgae grown in mass culture

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Abstract

Three species of microalgae were grown in mass culture to investigate the influence of culture technique and growth phase on the production of 20:5(n-3) and 22:6(n-3). These polyunsaturated fatty acids (PUFA) are considered to be essential in many marine animals diets for high growth and survival rates. The species of microalgae examined were *Nannochloropsis oculata*, *Pavlova lutheri* and *Isochrysis* sp. (clone T.Iso). All batch cultures (logarithmic and stationary phase) and semi-continuous cultures (logarithmic phase) examined contained high levels of the long-chain (n-3) PUFA, but production could be maximised by harvesting at specific times and growth phases. Maximum cellular content (pg cell^{-1}) of long-chain PUFA was found in logarithmic phase batch cultures of *N. oculata* and in stationary phase cultures of *P. lutheri*. The cellular content of PUFA in cultures of *Isochrysis* sp. did not change significantly with culture technique or growth phase. Alternatively, stationary phase cultures of all three species showed increased proportions (%) and cellular contents of triacylglycerols, and saturated and monounsaturated fatty acids with correspondingly decreased proportions of polar lipids and most PUFA relative to logarithmic phase cultures. The exception was the proportion and cellular content of 22:6(n-3) in *P. lutheri* which increased with triacylglycerol content. The mass of long-chain (n-3) PUFA per volume of culture was significantly higher in stationary phase cultures due to the higher cell counts per volume. These findings indicate that the opportunity exists to maximise PUFA production by microalgae with the potential to improve animal growth and reduce production costs in mariculture operations and may be of use in the large scale culture and harvesting of microalgae for the biotechnology industry.

Introduction

It has yet to be shown conclusively that marine animals can synthesise (n-3) and (n-6) polyunsaturated fatty acids (PUFA) *de novo*, although saturated and monoenoic fatty acids can be synthesised. In some studies, radiolabel from dietary ^{14}C-palmitic acid (16:0) was incorporated into long-chain C_{20} and C_{22} PUFA in species of crustaceans and fish, although it is unclear whether this was *de novo* synthesis, or incorporation of labelled acetate units (from the labelled 16:0) by chain elongation of shorter chain PUFA (Morris & Sargent, 1973; Jones *et al.*, 1979; Kanazawa *et al.*, 1980). Marine animals can produce C_{20} and C_{22} PUFA from dietary shorter chain PUFA but the desaturation and elongation steps are inefficient (Jones *et al.*, 1979; Kanazawa *et al.*,

1979; Kanazawa *et al.*, 1980). Because marine animals require 20:5(n-3) and 22:6(n-3) in excess of the amounts they can produce from shorter chain PUFA (e.g. Jones *et al.*, 1979), they must obtain these 'essential' long-chain PUFA from dietary sources. Requirements for either or both fatty acids may be species-specific (Helm, 1977; Helm & Laing, 1987).

Species of microalgae from the class Chlorophyceae, especially those of the genus *Chlorella*, are generally deficient in the C_{20} and C_{22} PUFA and have long been considered unsatisfactory feeds for mariculture animals [Dunstan *et al.*, (1992) and references therein]. Alternatively, many other species of microalgae contain high proportions of 20:5(n-3) and/or 22:6(n-3) (Volkman *et al.*, 1989) and marine animals fed such PUFA-rich microalgae maintain better growth in feeding trials than when fed similar diets lacking these PUFA (Langdon & Waldock, 1981; Enright *et al.*, 1986). The larval and juvenile animals which do not feed directly on microalgae (e.g. most fish and crustaceans) can be fed animals such as zooplankton which have been previously 'enriched' (i.e. fed) with 20:5(n-3) and 22:6(n-3) rich microalgae or a suitable substitute. Because the mass culture of microalgae for feeding to intensively reared animals is expensive and time consuming, it is important that the PUFA content of microalgae is maximised.

Batch cultures of microalgae grow logarithmically with time (logarithmic phase), until cell division rates decrease due to either depletion of a specific nutrient or nutrients or to a build up of growth limiting metabolites in the media. High cell counts per volume may also cause small decreases in division rates due to self-shading. Thus, the microalgal culture conditions are gradually and continually changing with time. When cell division rates approach zero due to growth limiting culture conditions, the static cultures are said to be in stationary phase. Batch cultures are usually grown to a certain age and harvested in their entirety (*i.e.* as a 'batch'), at late-logarithmic or stationary phase. To maintain high cellular division rates, cultures can be maintained in logarithmic phase by regular cropping of a portion of the culture and replenishment with fresh culture medium. This technique of semi-continuous harvesting is commonly used in the mariculture industry to produce a regular supply of microalgae for food and relative to batch cultures, usually results in an increased biomass yield per day (averaged over the life of the culture).

Lipid class and fatty acid compositions of microalgal cells at the different growth phases (logarithmic and stationary) can differ significantly (Emdadi & Berland, 1989; Hodgson *et al.*, 1991), and can change with variations in culture conditions e.g. nutrient status, temperature, salinity, pH, photoperiod, light intensity and light quality (reviewed by Yongmanitchai & Ward, 1989; Roessler, 1990). Thus, manipulation of culture conditions and harvesting at specific growth phases may enable the lipid and PUFA composition of microalgal cultures to be tailored for specific purposes.

In the present study, three species of microalgae used widely as feeds in the mariculture industry were selected and grown in mass culture. These were the eustigmatophyte *Nannochloropsis oculata* which has high concentrations of 20:5(n-3) (Maruyama *et al.*, 1986), the prymnesiophyte *Isochrysis* sp. (clone T.Iso) with high levels of 22:6(n-3) (Ben-Amotz *et al.*, 1985; Volkman *et al.*, 1989) and the prymnesiophyte *Pavlova lutheri* which has high levels of both of these PUFA (Volkman *et al.*, 1989; 1991). The effects of different culture techniques (batch and semi-continuous) and growth phase (logarithmic and stationary) on the lipid class and fatty acid composition in these three species were examined.

Materials and methods

Microalgal cultures

Cultures of *Nannochloropsis oculata* (Droop) Green (CS-179), *Isochrysis* sp. (Tahitian strain, clone T.Iso (CS-177) and *Pavlova lutheri* (Droop) Green (CS-182) were obtained from the CSIRO Algal Culture Collection (Jeffrey, 1980). For each species, two sets of duplicate 100 litre polyethylene bags containing 85 litres of f/2 media (using 0.2 μm membrane-filtered seawater; Lewis *et al.*,

1988) were inoculated from 1.2 litre axenic starter cultures. Cultures were aerated at a rate of 20 ml min^{-1} with food-grade air supplemented with CO_2 (0.5% by volume) and grown with a 12:12 h light:dark photoperiod (100 μmol photon $m^{-2} s^{-1}$) under controlled temperature (22 ± 2 °C) at Pipeclay Lagoon, Tasmania, Australia. Due to significant diurnal changes in the lipid class compositions which can occur (Sukenik & Carmeli, 1990), samples for biochemical analysis were taken at the same time of day (1000 h to 1200 h). Samples were taken initially from each bag at day 9 (logarithmic phase batch cultures). One set of duplicate bag cultures of each species was grown to stationary phase as batch cultures and sampled at day 23. Cell counts of stationary phase cultures ranged between $6–9 \times 10^6$ cells ml^{-1} for *Isochrysis* sp. $4.5–5.5 \times 10^6$ cells ml^{-1} for *P. lutheri* and $1.5–3.3 \times 10^7$ cells ml^{-1} for *N. oculata*. After day 9, the other set of duplicate bag cultures for each species was harvested [for feeding to juvenile oysters; *Crassostrea gigas* (Thunberg)] every second day (semi-continuously), by removing 15% of the culture volume and replenishing with fresh media to maintain the cultures in logarithmic phase. Because cell counts per volume in semi-continuous cultures of *N. oculata* and *P. lutheri* kept increasing at this harvest rate, to maintain a constant cell count, the volumes removed from these cultures were increased to 20% at days 15 and 17 respectively. These harvesting regimes maintained the cell counts at between $3–5 \times 10^6$ cells ml^{-1} for *Isochrysis* sp., $2–4 \times 10^6$ cells ml^{-1} for *P. lutheri* and $1–2.5 \times 10^7$ cells ml^{-1} for *N. oculata* which were significantly lower than the stationary phase cultures. These duplicate bags were subsequently sampled twice during the semi-continuous harvesting regime for biochemical analysis, on days 15 and 31 (day 30 for *P. lutheri*). After day 30, the semi-continuous harvesting was ceased and the *P. lutheri* cultures were grown to stationary phase and sampled at day 40.

Cell counts were made every day for the duration of the experiment; the average cell counts at sampling for biochemical analysis are shown in Table 1. Growth curves based on cell counts, including details of the semi-continuous harvesting and sampling regimes and results of cellular dry weight, amino acid, protein, carbohydrate and chlorophyll *a* analyses will be presented elsewhere.

Lipid extraction and fractionation

Cells were harvested from 200 ml of each culture by filtering through 47 mm diameter glass-fibre filters (Whatman GF/C). The filters were stored in liquid nitrogen prior to lipid analysis. The cells were extracted with chloroform-methanol-water (1:2:0.8, v/v/v; 5×8 ml for *P. lutheri* and *Isochrysis* sp., 7×8 ml for *N. oculata*; Bligh & Dyer, 1959) as outlined in Dunstan *et al.* (1992). Lipid extracts were stored in chloroform at −20 °C under nitrogen. The lipid class distributions were determined by analysing an aliquot of the lipid extract on Chromarod S-III silica rods with an Iatroscan Mk III TH-10 TLC-FID analyser (Iatron Laboratories, Japan). The solvent system used for the lipid separation was hexane-diethyl ether-acetic acid (60:17:0.2, v/v/v).

Fatty acid methyl esters (FAME) were formed by transesterification of a second aliquot of the solvent extract, with methanol-chloroform-HCl (10:1:1, v/v/v; 3 ml) at 80 °C for 2 h. After addition of 1 ml of Milli-Q water, the FAME were extracted with hexane-chloroform (4:1, v/v; 3×3 ml). After addition of heptadecanoic acid FAME as an internal standard, FAME were analysed using a polar Supelcowax 10, fused-silica column (60 m × 0.32 mm i.d., Supelco) in a Hewlett Packard 5890 gas chromatograph. FAME were further analysed with a non-polar methyl silicone (HP-1) fused-silica capillary column (50 m × 0.32 mm i.d., Hewlett Packard) in a Varian 3410 gas chromatograph with 8100 autosampler. Gas chromatographic conditions are given in Dunstan *et al.* (1992). Peak areas were quantified using DAPA software (Kalamunda, Western Australia). FAME were identified from retention index data on both polar and non-polar columns and confirmed from mass spectra obtained with an Hewlett-Packard 5970B GC-MSD system (Volkman *et al.*, 1989).

Fatty acids are designated as X:Y(n-z) where 'X' is the number of carbon atoms (as it is with the C_x notation), 'Y' is the number of double-bonds and 'z' is the position of the ultimate double-bond from the terminal methyl group. The prefix *i* indicates an *iso* methyl branch. The suffix *t* indicates *trans* geometry, double-bonds in fatty acids without this suffix are of *cis* geometry. FAME are fatty acid methyl esters, while PUFA is an abbreviation for polyunsaturated fatty acids. Double-bonds in PUFA are separated by a methylene group.

Results

For the three species of microalgae examined, the proportions (%) of the major lipid classes and fatty acids are presented in Table 1 and Table 2 respectively. The cellular content (pg cell^{-1}) of the major lipid classes and of 20:5(n-3) & 22:6(n-3) are presented in Figs 1a–1c and Figs 2a–2c respectively. Data on the mass of these two (n-3) PUFA per culture volume (in mg l^{-1}) have been included in Table 2. The lipid class and fatty acid compositional data in Tables 1 and 2 for the day 9 logarithmic phase batch cultures are the average of all four replicate cultures per species, (three in the case of *P. lutheri*). All other data presented (including the day 9 logarithmic phase batch cultures in the Figures) are the average of duplicate cultures except for the stationary phase culture of *P. lutheri* (single culture only) and only one of the duplicate cultures of stationary phase *N. oculata* was sampled at day 17. Ranges are provided for the major lipid classes (polar lipids, triacylglycerols and methyl alkenones; Fig. 1) and fatty acids (Fig. 2), and for total cellular lipid content (Table 1) where two or more samples were averaged, to give an indication of the limited spread of the data.

Lipid class compositions

(a) Logarithmic phase batch cultures

Logarithmic phase batch cultures of all three species of microalgae contained high proportions of polar lipid (83–90% of total lipid), with smaller amounts of 4-desmethyl sterols (1–4%) and triacylglycerols (trace-2%) (Table 1). These mass cultures of *P. lutheri* also had low proportions of compounds tentatively identified as steroidal diols (5%) and 4-methyl sterols (1%) as found in small scale batch cultures of this species (Volkman *et al.*, 1989, 1991). *Isochrysis* sp. had significant proportions of long-chain *trans*-unsaturated methyl (11%) and ethyl (4%) alkenones (ketones) in agreement with previous analyses (Volkman *et al.*, 1989).

(b) Semi-continuous cultures (logarithmic phase)

The cellular content of polar lipid in logarithmic phase semi-continuous cultures of *N. oculata* 0.4 pg cell^{-1}) was significantly lower than in the younger, logarithmic phase batch cultures (0.8 pg-cell^{-1}), the cellular content of the other lipid classes did not change significantly [Fig. 1a(i)]. Logarithmic phase semi-continuous cultures of *P. lutheri* (days 15 & 30) and *Isochrysis* sp. (days 15 & 31) had elevated cellular contents of triacylglycerols (0.3 & 0.1 and 0.6 & 0.3 pg cell^{-1} respectively), relative to the day 9 logarithmic phase batch cultures [trace and 0.03 pg cell^{-1} respectively; Figs 1b(i) & 1c(i)]. The cellular contents of methyl alkenones were higher in the logarithmic phase semi-continuous cultures of *Isochrysis* sp. compared with the logarithmic phase batch cultures (0.8 cf. 0.5 pg cell^{-1}). The abundance of the other lipid classes did not vary significantly in semi-continuous cultures of *P. lutheri* and *Isochrysis* sp. [Figs 1b(i) & 1c(i)].

(c) Stationary phase cultures

Cellular contents of triacylglycerols were significantly higher in all stationary phase cultures relative to logarithmic phase batch cultures [Figs 1a(ii)–1c(ii)]. As with logarithmic phase semi-continuous cultures of *N. oculata*, the amount of polar lipid in stationary phase cultures of this species (0.5–0.6 pg cell^{-1}) was significantly lower than in logarithmic phase batch cultures (0.8 pg cell^{-1}). The cellular contents of 4-methyl sterols, 4-desmethyl sterols and steroidal diols increased in stationary phase cultures of

Table 1. Proportions (%) of major solvent-extractable lipid classes and total cellular lipid content (pg cell^{-1}) in three species of microalgae grown in mass culture, sampled at different growth phases and cultured by different techniques.

Culture phase	Culture technique	Culture age (days)	Number of replicate cultures	Average cell count at sampling ($\times 10^6$ cells ml^{-1})	Lipid class (%)[1]									Total cellular lipid content[2] (pg cell^{-1} ± range)
					PL	SD	ST	4ME	FFA	TG	HC	MK	EK	
Nannochloropsis oculata														
Logarithmic	Batch	9	4	13.4	90	–	3.9	–	2.4	1.9	1.4	–	–	0.9 ± 0.10
Logarithmic	Semicontinuous	15	2	21.3	83	–	5.7	–	2.1	7.0	2.2	–	–	0.5 ± 0.14
Logarithmic	Semicontinuous	31	2	21.9	85	–	5.9	–	3.1	3.0	2.5	–	–	0.5 ± 0.04
Stationary	Batch	17	1	–	71	–	5.3	–	tr[3]	22	2.2	–	–	0.7
Stationary	Batch	23	2	24.3	51	–	4.6	–	0.5	41	3.1	–	–	1.1 ± 0.05
Pavlova lutheri														
Logarithmic	Batch	9	3	2.33	88	4.7	2.9	1.4	3.1	tr	–	–	–	5.0 ± 0.74
Logarithmic	Semicontinuous	15	2	3.72	84	5.4	2.9	1.5	1.2	4.9	–	–	–	5.3 ± 0.66
Logarithmic	Semicontinuous	30	2	2.97	87	5.2	2.5	1.5	1.5	2.4	–	–	–	5.1 ± 0.64
Stationary	Batch	40	2	3.54	50	5.9	5.0	2.0	1.0	35	0.5	–	–	8.2 ± 0.19
Stationary	Batch	23	1	5.06	57	6.2	4.5	2.5	1.2	28	0.7	–	–	7.0
Isochrysis sp.														
Logarithmic	Batch	9	4	3.53	83	–	1.2	–	–	0.6	–	11	3.8	4.8 ± 0.54
Logarithmic	Semicontinuous	15	2	4.07	72	–	0.7	–	–	11	–	14	3.2	6.1 ± 0.73
Logarithmic	Semicontinuous	31	2	4.42	74	–	0.8	–	–	4.8	–	16	4.2	4.9 ± 0.05
Stationary	Batch	23	2	7.25	54	–	0.8	–	–	20	–	22	4.1	7.0 ± 0.92

[1] PL, polar lipids and chlorophylls; SD, steroidal diols (tentative identification); ST, 4-desmethyl sterols; 4ME, 4-methyl sterols; FFA, free fatty acids; TG, triacylglycerols; HC, hydrocarbons; MK, C_{37}–C_{38} methyl ketones; EK, C_{38}–C_{39} ethyl ketones.

[2] Other minor components (less than 0.7%) not included in the Table are included in the total.

[3] Less than 0.5%.

Table 2. Proportions (%) of the major fatty acids, total cellular content of fatty acids (pg cell^{-1}) and production of "essential" PUFA per litre of culture in three species of microalgae grown in mass culture, sampled at different growth phases and cultured by different techniques.

	Nannochloropsis oculata					*Pavlova lutheri*					*Isochrysis* sp.			
	Logarithmic			Stationary		Logarithmic			Stationary		Logarithmic			Stationary
	Batch	Semicontinuous		Batch		Batch	Semicontinuous		Batch		Batch	Semicontinuous		Batch
Age days	9	15	31	17	23	9	15	30	40	23	9	15	31	23
replicates	4	2	2	1	2	3	2	2	2	1	4	2	2	2
Saturated Fatty Acids														
14:0	5.0	5.4	4.8	5.8	5.4	8.8	9.2	10.0	7.9	8.1	18.4	16.6	13.9	16.0
*i*15:0	0.3	0.5	0.5	0.1	tr[1]	0.1	tr	–[2]	–	0.1	tr	–	–	0.1
15:0	0.3	0.3	0.3	0.4	0.4	0.2	0.2	0.2	0.3	0.2	0.4	0.3	0.2	0.3
16:0	16.4	22.7	20.3	27.7	33.3	17.3	21.4	20.8	23.4	22.8	9.3	11.5	12.6	14.1
18:0	0.3	0.4	0.3	0.4	0.6	0.3	0.3	0.2	0.3	0.3	0.1	0.2	0.1	0.4
Subtotal	22.3	29.3	26.2	34.4	39.7	26.7	31.1	31.2	31.9	31.5	28.2	28.6	26.8	30.9
Monoenoic Fatty Acids														
16:1 (n-7)	21.8	19.7	21.2	19.6	24.4	14.4	18.9	19.9	23.0	20.9	5.0	2.9	3.2	2.9
16:1 (n-5)	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.2	tr	–	–	tr
16:1 (n-13)*t*	1.0	0.8	0.9	0.4	0.2	–	–	–	–	–	0.1	tr	0.1	tr
17:1 (n-8)	0.2	0.1	0.2	0.1	0.2	–	–	–	–	–	0.2	0.1	0.2	0.1
18:1 (n-9)	2.1	5.3	4.1	9.0	11.9	0.6	0.6	0.5	1.3	0.8	7.6	16.7	15.1	25.6
18:1 (n-7)	0.6	0.6	0.5	0.4	0.5	2.4	3.2	3.3	3.3	3.4	2.3	0.9	1.7	1.4
Subtotal	25.8	26.6	27.0	29.6	37.3	17.5	22.9	23.8	27.8	25.3	15.2	20.6	20.3	30.0
Polyenoic Fatty Acids														
16:2 (n-7)	1.0	0.5	0.4	0.2	0.1	2.1	0.7	1.1	0.7	0.5	1.4	0.8	0.3	0.6
16:2 (n-4)	0.5	0.2	0.1	0.1	0.1	0.7	0.6	0.7	0.5	0.6	0.6	0.5	0.5	0.5
18:2 (n-6)	1.7	1.7	1.8	1.3	0.9	0.6	0.5	0.4	0.8	0.5	3.7	4.5	4.5	3.7
18:3 (n-6)	0.9	0.5	1.0	0.4	0.4	0.5	0.2	0.2	0.2	0.1	2.0	1.4	0.7	0.3
18:3 (n-3)	0.1	0.1	tr	0.1	tr	2.1	1.1	1.1	0.6	0.6	5.7	4.7	5.8	3.9
18:4 (n-3)	–	–	–	–	–	10.9	7.9	6.6	4.9	6.2	26.8	24.2	25.4	17.1
18:5 (n-3)	–	–	–	–	–	–	–	–	–	–	2.5	1.4	0.3	0.7
20:4 (n-6)	6.1	6.7	7.4	6.3	3.9	0.7	0.6	0.6	0.6	0.5	0.1	tr	tr	0.1
20:5 (n-3)	39.8	33.4	34.7	25.9	16.4	27.6	23.1	22.9	17.0	17.7	0.8	0.6	0.6	0.5
22:5 (n-6)	–	–	–	–	–	0.9	1.1	0.9	1.5	1.6	1.4	1.9	2.3	1.4
22:5 (n-3)	–	–	–	–	–	–	0.1	0.1	0.8	0.9	tr	tr	0.1	–
22:6 (n-3)	–	–	–	–	–	7.9	8.1	7.9	9.2	11.2	9.5	9.0	10.2	6.8
Subtotal	50.1	43.1	45.4	34.3	21.8	54.0	44.0	42.5	36.8	40.4	54.5	49.0	50.7	35.6
Others[3]	1.8	1.0	1.4	1.7	1.2	1.8	2.0	2.5	3.5	2.8	2.1	1.8	2.2	3.5
TOTAL	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total cellular fatty acid content														
(pg cell^{-1})	0.45	0.34	0.28	0.43	0.75	2.27	2.04	2.01	3.63	3.56	2.04	2.30	1.70	2.99
PUFA production per litre of culture														
mg 20:5 (n-3)/l	2.45	2.42	2.12	3.63	2.95	1.45	1.75	1.37	2.20	3.19	0.05	0.06	0.04	0.10
mg 22:6 (n-3)/l	–	–	–	–	–	0.42	0.61	0.48	1.18	2.01	0.69	0.84	0.77	1.48

(1) Less than 0.05%.

(2) None detected.

(3) Sum of less abundant (less than 1%) and unidentified fatty acids.

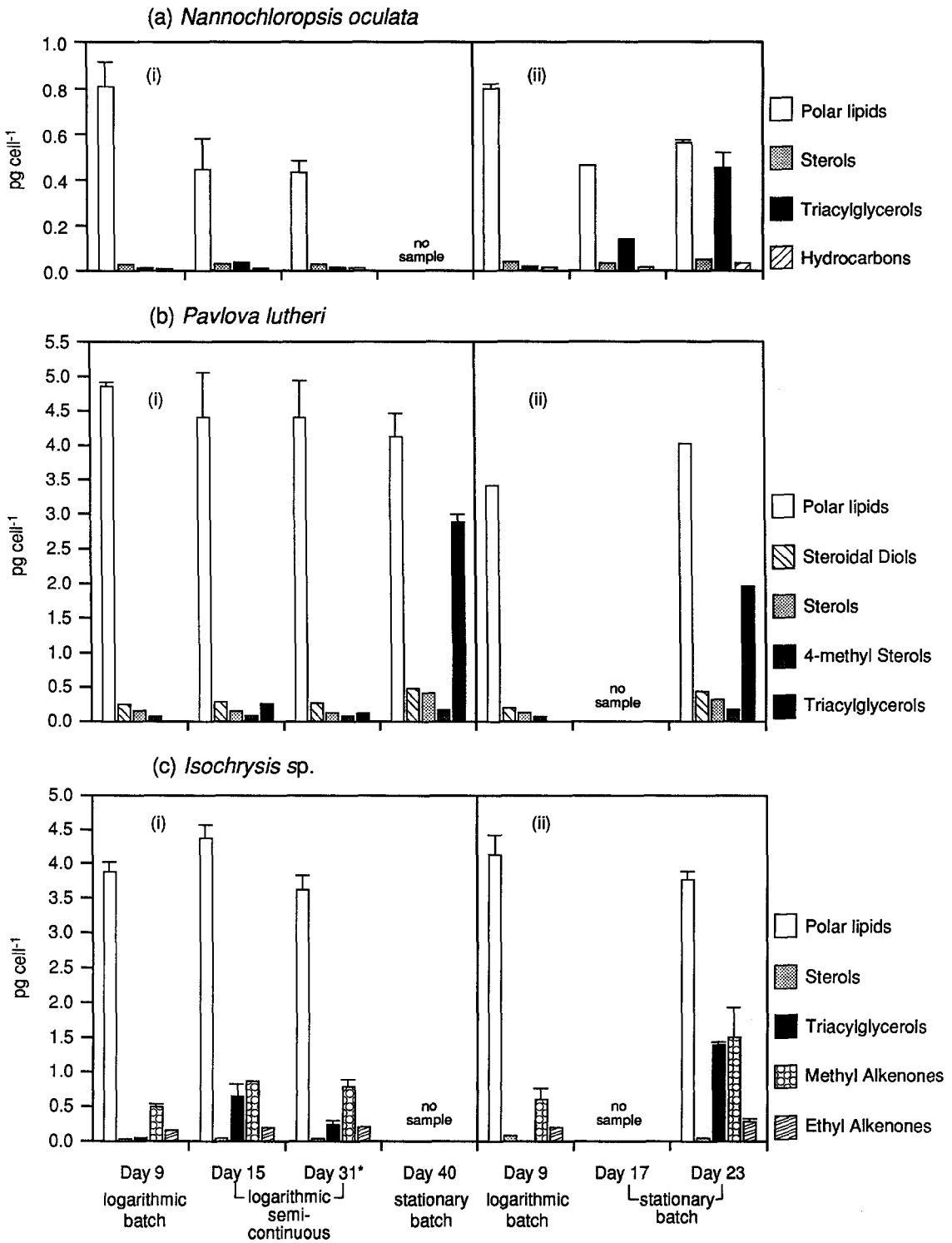

Fig. 1. Average cellular content (pg cell^{-1}) of lipid classes in duplicate bag cultures of (a) *Nannochloropsis oculata*, (b) *Pavlova lutheri* and (c) *Isochrysis* sp. (i) maintained in logarithmic phase by semi-continuous harvest and (ii) grown to stationary phase. Range bars are included on the major lipid classes, where date represent the average of two samples. (* day 30 for *P. lutheri*).

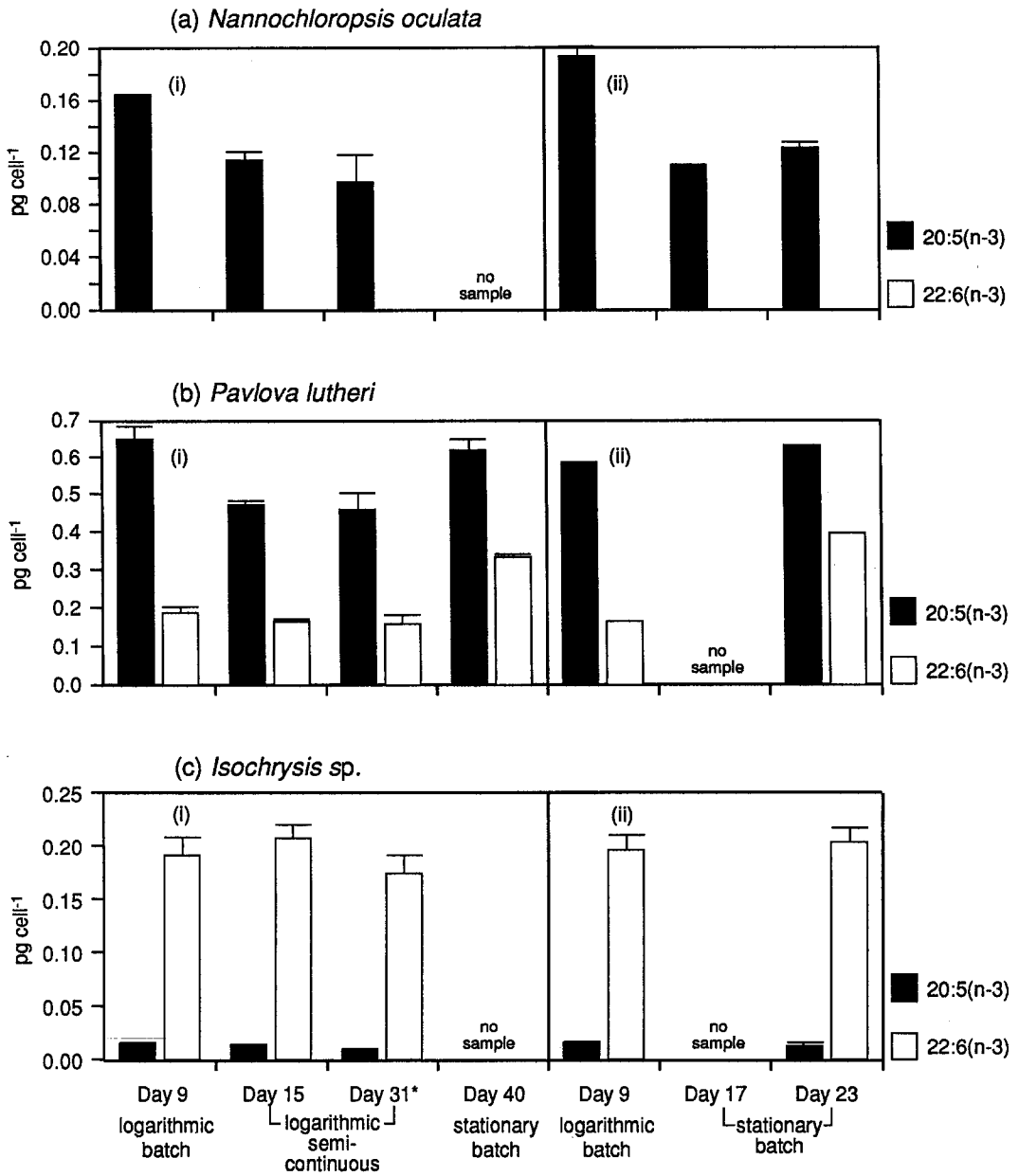

Fig. 2. Average cellular content (pg cell^{-1}) of 20:5(n-3) and 22:6(n-3) in duplicate bag cultures of (a) *Nannochloropsis oculata*, (b) *Pavlova lutheri* and (c) *Isochrysis* sp. (i) maintained in logarithmic phase by semi-continuous harvest and (ii) grown to stationary phase. Range bars are included where data represent the average of two samples. (* day 30 for *P. lutheri*).

P. lutheri relative to logarithmic phase batch cultures [Fig. 1b(ii)]. Cultures of *P. lutheri* maintained in logarithmic phase by semi-continuous harvesting for 30 days and then grown to stationary phase, had similar lipid class distributions to the culture grown directly to stationary phase in batch culture [Table 1, Fig. 1b(i & ii)]. The cellular contents of methyl and ethyl alkenones in *Isochrysis* sp. were significantly higher in stationary phase cultures (1.5 and 0.3 pg cell^{-1} respectively) relative to logarithmic phase batch cultures [0.6 and 0.2 pg cell^{-1} respectively; Fig. 1c(ii)].

Fatty acid compositions

(a) Logarithmic phase batch cultures

The major saturated fatty acids in the three species were 14:0 and 16:0. The major monoenoic fatty acids were 16:1(n-7) and/or 18:1(n-9). Generally 18:1(n-9) was more abundant than 18:1(n-7), although notable exceptions were the cultures of *P. lutheri* (Table 2) as previously reported for small scale batch cultures of this species (cited as *Monochrysis lutheri* in Ackman *et al.*, 1968). The very low amounts of branched chain fatty acids (the most abundant being *i*15:0, 0–0.5%, Table 2), indicate that the non-axenic mass cultures were not significantly contaminated with the bacteria which produce these fatty acids (Volkman *et al.*, 1989). All species of microalgae examined had high proportions of PUFA (20–55%), but the proportions of the individual fatty acids differed markedly between the three species and with growth phase (Table 2).

(b) Semi-continuous cultures (logarithmic phase)

In cultures maintained in logarithmic phase by semi-continuous harvesting, the proportions (%) of 16:0 and 18:1(n-9) increased in *Isochrysis* sp. and *N. oculata* as did the proportions of 16:0 and 16:1(n-7) in *P. lutheri*, (Table 2). Logarithmic phase semi-continuous cultures of *N. oculata* and *P. lutheri*, had decreased proportions and cellular contents (pg cell^{-1}) of most PUFA relative to logarithmic phase batch cultures [Table 2, e.g. Figs 2a(i)–2c(i)]. The exception was the proportion and cellular content of 22:6(n-3) in *P. lutheri*, which did not change significantly [Table 2, Fig. 2b(i)]. Logarithmic phase semi-continuous cultures of *Isochrysis* sp. showed little change in the cellular contents of 20:5(n-3) and 22:6(n-3) [Fig. 2c(i)] or the proportions of these and other PUFA (Table 2), relative to logarithmic phase batch cultures.

(c) Stationary phase cultures

Stationary phase cultures of all species had increased total cellular fatty acid content, significantly elevated proportions and cellular contents of saturated and monoenoic fatty acids and significantly reduced proportions of PUFA (Table 2). *N. oculata* had reduced contents of most PUFA per cell [e.g. Fig. 2a(ii)], whereas the cellular content of long chain (n-3) PUFA in *Isochrysis* sp. did not change significantly with culture phase [Fig. 2c(ii)]. In contrast, stationary phase cultures of *P. lutheri* (including the cultures grown to stationary phase after being harvested semi-continuously) had higher cellular contents of most fatty acids including C_{22} PUFA [especially 22:6(n-3) Fig. 2b(ii)]. The mass of 20:5(n-3) and 22:6(n-3) per culture volume (Table 2) was dependant on the cell counts (per volume) at sampling. Thus, stationary phase cultures (highest cell counts; Table 1) had significantly higher amounts of these (n-3) PUFA per litre of culture than logarithmic phase cultures.

Discussion

Lipid class and fatty acid compositions

The lipid class and fatty acid compositions of logarithmic phase batch cultures from the present study (Tables 1 & 2) were very similar to those found previously for small scale batch cultures of the same species examined at the same growth phase (Volkman *et al.*, 1989, 1991). Total cellular lipid content (Table 1) for *Pavlova lutheri* (5–8 pg cell^{-1}) was similar to reported values for the same species [4.6–7.5 pg cell^{-1} (Thompson *et al.*, 1990)]; whereas total cellular lipid content in *Isochrysis* sp. (5–7 pg cell^{-1}) was higher than reported elsewhere [2.6–5 pg cell^{-1} (Thompson *et al.*, 1990), 3–6 pg cell^{-1} (Sukenik & Wahnon, 1991)]. Our data for *N. oculata* (0.9–1.1 pg cell^{-1}) is low compared with this and other species of eustigmatophyte at similar growth phases during batch culture, and analysed using similar extraction techniques but different lipid quantitation methods; 1.3–2.8 pg cell^{-1} (daily variation, Sukenik & Carmeli, 1990) and 1.5–7 pg cell^{-1} (with culture age, Hodgson *et al.*, 1991).

The major lipid classes in microalgae are the polar lipids (mostly phospholipids and glycolip-

ids) which are common membrane components; and the triacylglycerols which are a reserve of fatty acids for cellular division, metabolic energy, membrane maintenance, synthesis and a variety of physiological uses. The relative amounts of each lipid class in microalgal cells can change considerably with variations in culture conditions (reviewed by Yongmanitchai & Ward, 1989; Roessler, 1990). If the light energy harnessed by cells is in excess of that needed for cell division and maintenance (as happens at stationary phase when cells are inhibited from dividing due to limiting culture conditions), this energy can be used to synthesise storage lipids (Roessler, 1990). The lipids produced are primarily stored as triacylglycerols and mostly synthesised *de novo* from CO_2 (Suen *et al.*, 1987), although a smaller proportion of the lipids produced may be converted from pre-existing non-lipid compounds (Roessler, 1990). When conditions improve, the triacylglycerols are readily available for the initial rapid cell division of logarithmic phase. Thus, the accumulation of triacylglycerols is usually coincident with stationary phase because both can be triggered by limiting culture conditions.

The accumulation of triacylglycerols in all three species examined in the present study, is clearly associated with the cells reaching stationary phase [Figs 1b(i), 1a(ii)–1c(ii)]. Previous work has shown similar accumulation of triacylglycerols in stationary phase cultures of *N. salina* and *P. lutheri* (Emdadi & Berland, 1989). Nutrient limitation has been shown to induce stationary phase in batch cultures of *Isochrysis* sp. (clone T.Iso) (Graske & van der Molen, 1992) and may well be the limiting culture condition responsible for the onset of this static phase in the present study. If the limiting culture condition is reduced nitrogen available for amino acid synthesis, the result can be (depending on the species) cells with proportionally less protein, and the tendency to store energy as carbohydrates and lipids (mostly triacylglycerol) (reviewed by Roessler, 1990). Nitrogen-limited batch cultures (which were early stationary phase) of *I. galbana* tended to accumulate carbohydrates with little change in lipid content (Harrison *et al.*, 1990; Sukenik and Wahnon, 1991), although severely limited cultures of the same species did accumulate triacylglycerols (Sukenik and Wahnon, 1991). Similar findings have been reported in nitrogen-limited cultures of diatoms (Shifrin & Chisholm, 1981 and references therein). The accumulation of triacylglycerols has also been recorded in actively growing cultures of diatoms due to reduced nutrient levels (Parrish & Wangersky, 1990; Lombardi & Wangersky, 1991) which may account for the slight increases in the levels of triacylglycerols in the logarithmic cultures of *P. lutheri* and *Isochrysis* sp. harvested semi-continuously [Figs 1b(i) & 1c(i)].

The distributions of fatty acids esterified to individual lipid classes differ significantly, with the triacylglycerols generally having proportionally more saturated and monoenoic fatty acids (Suen *et al.*, 1987), compared with polar lipid which generally have proportionally more PUFA (Piorreck *et al.*, 1984; Hodgson *et al.*, 1991; Sukenik & Wahnon, 1991). Because these lipid class fatty acid distributions differ, changes in the lipid class composition induced by variations in culture conditions and growth phase will result in changes in the total cellular fatty acid composition. Therefore, samples with high levels of triacylglycerols should contain relatively higher proportions (%) of saturated and monoenoic fatty acids and lower proportions of PUFA, as is evident in the stationary phase cultures of all three species examined in the present study (Table 2) and the work of others (Piorreck *et al.*, 1984; Suen *et al.*, 1987; Hodgson *et al.*, 1991). The increased proportion (Table 2) and cellular content of 22:6(n-3) [Figs 2b(i & ii)] in *P. lutheri* in stationary phase relative to logarithmic phase, indicates that this PUFA may make up a larger proportion of the triacylglycerol fatty acids than in the other species examined. Thus the production of this fatty acid by *P. lutheri* may be enhanced if cultures are grown to stationary phase (to maximise triacylglycerol content) prior to harvest.

Decreases in the proportions (%) of polar lipid in all stationary phase cultures relative to logarithmic phase batch cultures (Table 1) were primarily due to increases in the amounts of triacylglycerols [Figs 1b(i), 1a(ii)–1c(ii)]. Overall the

cellular content (pg cell^{-1}) of the major long-chain PUFA in each species [20:5(n-3) in *N. oculata* and *P. lutheri* and 22:6(n-3) in *Isochrysis* sp.] appears to be related to the cellular content of polar lipid (Figs 1a cf. 2a; 1b cf. 2b; 1c cf. 2c). This observation is probably due to the major long-chain PUFA in each species being predominantly resident on the polar lipids of the cellular membranes. Significant decreases in the cellular content of polar lipid and 20:5(n-3), in stationary batch and logarithmic phase semi-continuous cultures of *N. oculata* relative to the logarithmic phase batch cultures were evident (Figs 1a & 2a). Reduced polar lipid content of cells may be due to cellular membrane deterioration induced by nutrient deficiencies (Emdadi & Berland, 1989), e.g. phosphate limiting of cultures may reduce the cells ability to produce phospholipids for membranes. Also, reduced production of the protein-rich chloroplasts, resulting from reduced amino acid synthesis in nitrogen-limited cultures, may in turn result in a reduced need for the phospholipids and glycolipids associated with these organelles (Piorreck *et al.*, 1984). It is unlikely that such nutrient deficiencies caused the reduced cellular content of polar lipid in semi-continuously harvested cultures of *N. oculata* as there was no accompanying increase in triacylglycerols [Fig. 1a(i)]. Alternatively, the reduced cellular content of polar lipid and 20:5(n-3) and the 20–40% decrease in total cell mass in stationary phase and semi-continuous cultures of *N. oculata* (unpublished data) probably reflect the greater abundance of smaller cells in the older cultures.

Applications to mariculture and biotechnology

For mariculture operations feeding animals rations based on cell counts per volume, to minimise the number of microalgal cells needed to achieve the same PUFA ration per animal, maximum PUFA content per cell would be required. To achieve this in the species examined, logarithmic phase batch cultures of *N. oculata* (Fig. 2a) and stationary phase cultures of *P. lutheri* (Fig. 2b) should be harvested. Because there was little effect of growth phase or culture technique on the cellular contents of PUFA in *Isochrysis* sp., harvest time in this species would be less important with respect to cellular contents of PUFA (Fig. 2c). Due to significant diurnal variations in the production and turnover of triacylglycerols, glycolipids and fatty acids in some species of microalgae, PUFA content can further be optimised by harvesting at particular times of the day (e.g. Sukenik & Carmeli, 1990).

For mariculture and biotechnology operations in which the production of PUFA is measured and used based on culture volume, stationary phase cultures (with the highest cell counts per volume) produced significantly higher amounts of the (n-3) PUFA per litre of culture than logarithmic phase cultures (Table 2). It should be noted that the total yield of PUFA over the entire life of the culture needs to be considered. For example, combined harvests from the semi-continuous cultures (which can be sustained and cropped for several weeks), should yield more of the (n-3) PUFA in total, than a single cropping of the relatively lipid and (n-3) PUFA-rich stationary phase batch cultures. This is even though at any one time, the former contains lower amounts of these PUFA per culture volume than the latter.

If the lipid class to which fatty acids are esterified or the overall proportions or amounts of fatty acids in the dietary microalgae are considered to be nutritionally important to the species of animal being intensively reared (e.g. due to differential assimilation of fatty acids), the culture growth phase may determine optimum time for harvest. Harvesting any of the relatively lipid-rich stationary phase cultures examined, results in proportionally less polar lipid and PUFA in the microalgal cells due to the increased proportions of triacylglycerol and saturated and monoenoic fatty acids (Tables 1 & 2). The nutritional significance of which, should be evaluated for the particular animal being reared.

In conclusion, for each species of microalgae cultured, the different proportions, cellular contents and/or culture content (per volume) of (n-3) PUFA produced with culture type and phase needs to be seriously considered in conjunction

with the economics (incorporating productivity and cost per yield) and specific requirements of each mariculture or biotechnology operation.

Other lipids

Slight increases with culture age in the level of hydrocarbons in *N. oculata* (from 1.4% to 3.1% of total lipid) and *P. lutheri* (from 0 to 0.7% of total lipid; Table 1) are consistent with observations for a eustigmatophyte (cited as *Nannochloris* sp.) in nitrogen-limited induced stationary phase cultures (Ben-Amotz *et al.*, 1985). Emdadi & Berland (1989) found remarkably high concentrations of hydrocarbons in lag-phase cultures of *P. lutheri* (48% of total lipid) and *N. salina* (78% of total lipid). These hydrocarbons are the end products of photosynthesis, which are accumulated in slow growing or stressed cells (Ben-Amotz *et al.*, 1985; Emdadi & Berland, 1989). When such old stationary phase cultures are transferred to non-limiting culture conditions, after a brief 'lag' phase, the cells contain hydrocarbons in amounts similar to values found in the present study for logarithmic phase cultures (Ben-Amotz *et al.*, 1985).

Each of the species examined is known to produce significant amounts of unusual lipids. *N. oculata* produces long-chain (C_{30} & C_{32}) alkyl diols and unsaturated alcohols (these polar lipids represent approximately 10% of the total lipid; Volkman *et al.*, 1991). *P. lutheri* produces 4-methyl sterols (1.4–2.5% of total lipid) and compounds tentatively identified as steroidal diols (4.7–12.5% of total lipid) (Table 1; Ballantine *et al.*, 1979; Volkman *et al.*, 1989, 1991) and *Isochrysis* sp. produces long-chain fatty acids (C_{36}), hydrocarbons (C_{37}-C_{38}) and methyl (C_{37}-C_{38}) and ethyl (C_{38}-C_{39}) alkenones (15–25% of total lipid; Table 1 & unpublished data).

Ballantine *et al.* (1979) did not detect the compounds assigned as steroidal diols in logarithmic phase cultures of *P. lutheri* (cited as *Monochrysis lutheri*) although they were detected in stationary phase cultures. They suggested that these compounds might be produced from oxidation of the sterols. Our data show that the compounds are present in both logarithmic and stationary phase cultures of *P. lutheri*. However, the proportions and total cellular contents of 4-methyl sterols and 'steroidal diols' were significantly greater in stationary phase cultures of *P. lutheri* [Table 1 & Fig. 1b(ii)], so the difference in the results may simply reflect the detection limits of the Ballantine *et al.* (1979) study.

The proportion and total cellular content of alkenones in *Isochrysis* sp. was significantly higher in logarithmic phase semi-continuous and stationary phase cultures than logarithmic phase batch cultures (Table 1 & Fig. 1c). Similarly, Ben-Amotz *et al.* (1985) noted that the C_{37} alkenone in nitrogen-deficient cultures of *Isochrysis* sp. increased in concentration. The role of these compounds in *Isochrysis* sp. is not known, but it seems likely that they are associated with membrane structure. These alkenones are significant components in stationary phase cultures of *Isochrysis* (25.6% of total lipid) which must be taken into account when calculating fatty acid content from total lipid (cf. López Alonso *et al.*, 1992). Even in species which do not contain alkenones, the assumption that the total lipid content is synonymous with the total fatty acid content is clearly erroneous (Totals in Tables 1 cf. 2; Seto *et al.*, 1984; Dunstan *et al.*, 1992).

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References

Ackman RG, Tocher CS, McLachlan J (1968) Marine phytoplankter fatty acids. J. Fish. Res. Bd Canada 25: 1603–1620.

Ballantine JA, Lavis A, Morris RJ (1979) Sterols of the phytoplankton-effects of illumination and growth stage. Phytochemistry 18: 1459–1466.

Ben-Amotz A, Tornabene TG, Thomas WH (1985) Chemical profiles of selected species of microalgae with emphasis on lipids. J. Phycol. 21: 72–81.

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 912–917.

Dunstan GA, Volkman JK, Jeffrey SW, Barrett SM (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae 2. Lipid classes and fatty acids. J. exp. mar. Biol. Ecol. 161: 115–134.

Emdadi D, Berland B (1989) Variation in lipid class composition during batch growth of *Nannochloropsis salina* and *Pavlova lutheri*. Mar. Chem. 26: 215–225.

Enright CT, Newkirk GF, Craigie JS, Castell JD (1986) Evaluation of phytoplankton as diets for juvenile *Ostrea edulis* L. J. exp. mar. Biol. Ecol. 96: 1–13.

Graske J, van der Molen M (1992) Effects of nutrient concentration on the growth of Tahitian *Isochrysis*. Austasia Aquaculture 6: 43–47.

Harrison PJ, Thompson PA, Calderwood GS (1990) Effects of nutrient and light limitation on the biochemical composition of phytoplankton. J. appl. Phycol. 2: 45–56.

Helm MM (1977) Mixed algal feeding of *Ostrea edulis* larvae with *Isochrysis galbana* and *Tetraselmis suecica*. J. mar. biol. Ass. U.K. 57: 1019–1029.

Helm MM, Laing I (1987) Preliminary observations on the nutritional value of Tahiti *Isochrysis* to bivalve larvae. Aquaculture 62: 281–288.

Hodgson PA, Henderson RJ, Sargent JR, Leftley JW (1991) Patterns of variation in the lipid class and fatty acid composition of *Nannochloropsis oculata* (Eustigmatophyceae) during batch culture I. The growth cycle. J. appl. Phycol. 3: 169–181.

Jeffrey SW (1980) Cultivating unicellular marine plants. In: CSIRO Division of Fisheries and Oceanography, Annual report (1977–1979), 22–43.

Jones DA, Kanazawa A, Ono K (1979) Studies on the nutritional requirements of the larval stages of *Penaeus japonicus* using microencapsulated diets. Mar. Biol. 54: 261–267.

Kanazawa A, Teshima S, Imai K (1980) Biosynthesis of fatty acids in *Tilapia zillii* and the puffer fish. Mem. Fac. Fish. Kagoshima Univ. 29: 313–318.

Kanazawa A, Teshima S, Kazuo O (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. 63B: 295–298.

Langdon CJ, Waldock MJ (1981) The effect of algal and artificial diets on the growth and fatty acid composition of *Crassostrea gigas* spat. J. mar. biol. Assoc. U.K. 61: 431–448.

Lewis TE, Garland CD, O'Brien TD, Fraser MI, Tong PA, Ward C, Dix TG, McMeekin TA (1988) The use of 0.2-μm membrane-filtered seawater for improved control of bacterial levels in microalgal cultures fed to larval Pacific oysters (*Crassostrea gigas*). Aquaculture 69: 241–251.

Lombardi AT, Wangersky PJ (1991) Influence of phosphorus and silicon on lipid class production by the marine diatom *Chaetoceros gracilis* grown in turbidostat cage cultures. Mar. Ecol. Prog. Ser. 77: 39–47.

López Alonso D, Molina Grima E, Sánchez Pérez E, García Sánchez JL, García Camacho F (1992) Isolation of clones of *Isochrysis galbana* rich in eicosapentaenoic acid. Aquaculture 102: 363–371.

Maruyama I, Nakamura T, Matsubayashi T, Ando Y, Maeda T (1986) Identification of the alga known as 'marine *Chlorella*' as a member of the Eustigmatophyceae. Jap. J. Phycol. 34: 319–325.

Morris RJ, Sargent JR (1973) Studies on the lipid metabolism of some oceanic crustaceans. Mar. Biol. 22: 77–83.

Parrish CC, Wangersky PJ (1990) Growth and lipid class composition of the marine diatom, *Chaetoceros gracilis*, in laboratory and mass culture turbidostats. J. Plankton Res. 12: 1011–1021.

Piorreck M, Baasch K, Pohl P (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue green algae under different nitrogen regimes. Phytochemistry 23: 207–216.

Roessler PG (1990) Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. J. Phycol. 26: 393–399.

Seto A, Wang HL, Hesseltine CW (1984) Culture conditions affect eicosapentaenoic acid content of *Chlorella minutissima*. J. am. Oil Chem. Soc. 61: 892–894.

Shifrin NS, Chisholm SW (1981) Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. J. Phycol. 17: 374–384.

Suen Y, Hubbard JS, Holzer G, Tornabene TG (1987) Total lipid production of the green alga *Nannochloropsis* sp. QII under different nitrogen regimes. J. Phycol. 23: 289–296.

Sukenik A, Carmeli Y (1990) Lipid synthesis and fatty acid composition in *Nannochloropsis* sp. (Eustigmatophyceae) grown in a light-dark cycle. J. Phycol. 26: 463–469.

Sukenik A, Wahnon R (1991) Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*. Aquaculture 97: 61–72.

Thompson PA, Harrison PJ, Whyte JNC (1990) Influence of irradiance on the fatty acid composition of phytoplankton. J. Phycol. 26: 278–288.

Volkman JK, Dunstan GA, Jeffrey SW, Kearney PS (1991) Fatty acids from microalgae of the genus *Pavlova*. Phytochemistry 30: 1855–1859.

Volkman JK, Jeffrey SW, Nichols PD, Rogers GI, Garland CD (1989) Fatty acid and lipid composition of 10 species of microalgae used in mariculture. J. exp. mar. Biol. Ecol. 128: 219–240.

Yongmanitchai W, Ward OP (1989) Omega-3 fatty acids: alternative sources of production. Process Biochem. August: 117–125.