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The effect of growth phase on the lipid class, fatty acid and sterol composition in the marine dinoflagellate, *Gymnodinium* sp. in batch culture

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Abstract

We have studied the effects of growth phase on the lipid composition in batch cultures of *Gymnodinium* sp. CS-380/3 over 43 days of culturing. The lipid content increased two fold, from late logarithmic (day 6) to linear growth phase (day 22) then decreased at stationary phase (day 43) while the lipid yield (mg l^{-1}) increased 30-fold from day 6 to 30 mg l^{-1} at day 43. Changes in fatty acid content mirrored those observed for the total lipid, while the sterol content continued to increase with culture age through to stationary phase. The largest changes occurred in the lipid classes, especially the polar lipids and triacylglycerols (oil). The proportion of triacylglycerols increased from 8% (of total lipids) at day 6 to 30% at day 43, with a concomitant decrease in the polar lipid fraction. The proportions of 16:0 and DHA [22:6(n-3)] increased while those of 18:5(n-3) and EPA [20:5(n-3)] decreased with increasing culture age. The proportion of the major sterol, dinosterol, decreased from 41% (day 6) to 29% (day 43), while the major dinostanol epimer (23*R*,24*R*) increased from 33% (day 6) to 38% (day 22). Despite small changes in the proportion of the main sterols, the same sterols were present at all stages of growth, indicating their value as a chemotaxonomic tool for distinguishing between strains within the same genus. Growth phase could be a useful variable for optimising the oil and DHA content with potential for aquaculture feeds and a source of DHA-rich oils for nutraceuticals.

Keywords: Alkylation; Chemotaxonomy; Desaturation; Dinophyceae; Fatty acids; Growth phase; *Gymnodinium* sp.; Lipids; Sterols; Triacylglycerols

1. Introduction

The lipid composition of microalgae, particularly the fatty acid profile, has been used as a general chemotaxonomic guide to characterize microalgal classes (e.g. Volkman, 1989). The lipid content, lipid class composition and the proportions of the various fatty acids in a microalga can vary and this has raised concerns about possible limitations in the use of lipids as chemotaxonomic markers. The fatty acids can be affected by a number of environmental or culturing variables such as growth phase (Dunstan et al., 1993; Brown et al., 1996; Zhu et al., 1997; Hatate et al., 1998), light intensity and photoperiod (Brown et al. 1996; Tzovenis et al., 1997), temperature (Thompson et al., 1992; Zhu et al., 1997; McLachlan et al., 1999), salinity (Xu and Beardall, 1996), carbon dioxide concentration (Pronina et al., 1998), nitrogen, phosphorus and $MgCl_2$ concentration (Dempster and Sommerfeld, 1998) and the intensity of UV-B irradiation (Skerratt et al., 1998).

The sterol profiles can consist of complex mixtures with a large diversity of sterol structures. Sterol profiles also lack commonality within the same algal class. For example, sterol compositions have been recently shown to vary quite considerably between different species of dinoflagellates and even within the same genus (Mansour et al., 1999b; Volkman et al., 1999). Sterol profiles have been considered to be more stable than the fatty acid distributions, and less likely to be affected by growth phase (Hallegraeff et al., 1991). However, quantitative analyses validating the stability of the sterol profile with growth phase are lacking and some data are contradictory. For example, major differences were reported in the types of sterols present in *Gymnodinium*

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sp. from the Adriatic Sea harvested at logarithmic and stationary phases (Piretti et al., 1997), whereas Hallegraeff et al. (1991) found that, in contrast to changes in fatty acid composition, growth phase had little effect on the sterol composition of *Gymnodinium catenatum*.

To address the possible effects of growth phase on the stability of the fatty acid and sterol compositions, we analysed the fatty acid and sterol compositions in cultures of the dinoflagellate *Gymnodinium* sp. CS-380/3 in batch culture to assess their use as chemotaxonomic tools. Another objective was to examine the effect of growth phase on lipid content and the proportion of triacylglycerols (oil), to see if growth phase is a factor which can be used to optimise the 22:6(n-3) (docosahexaenoic acid; DHA) and oil content for possible applications such as DHA-rich live feeds/supplements for the aquaculture industry and DHA-rich oils for the nutraceutical industry.

2. Results and discussion

2.1. Growth

Three distinct growth phases (Fogg, 1965) are shown on the growth curve (Fig. 1). The microalga underwent logarithmic growth from day 0 to day 6 with a mean specific growth rate of 0.28 d^{-1} (1 division every 3.5 days) and entered linear phase between day 7 and day 8 ($\mu = 0.28$ d^{-1}, days 6–7; $\mu = 0.20$ d^{-1}, days 6–8; and $\mu = 0.12$ d^{-1}, days 7–8). This was followed by a extended period of slow growth (arithmetic or linear growth phase) (Becker, 1994; Tredici, 1999), from day 8 to day 27 with an average specific growth rate of $\mu = 0.10$ d^{-1} (one division every 10 days) indicating growth limitation due to lack of gas exchange, nutrients or other factors. The onset of stationary phase occurred from day 27 to day 29 and stationary phase lasted from day 29 to day 43.

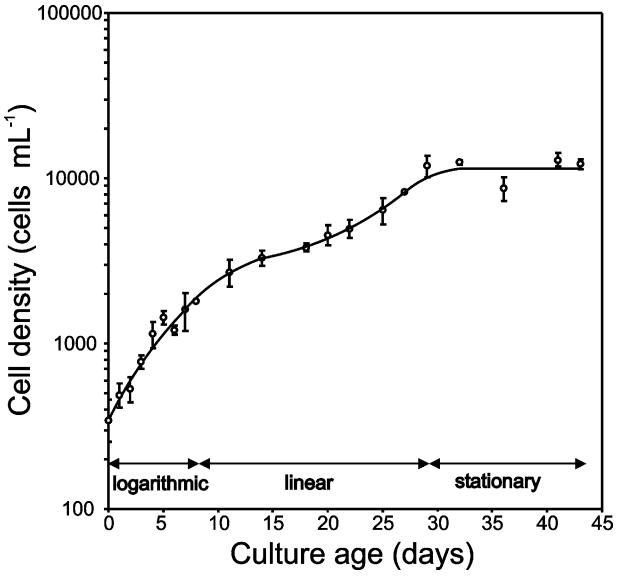

Fig. 1. The growth curve for *Gymnodinium* sp. CS-380/3. Logarithmic growth was between days 0 and 7 ($\mu = 0.28$ d^{-1}, 1 division every 3.5 days). The culture entered linear growth phase between day 7 and day 8 ($\mu = 0.12$ d^{-1}) and had extended linear growth from day 8 to 27 ($\mu = 0.10$ d^{-1}, 1 division every 10 days). The onset of stationary phase was at day 27 and continued to day 43. Cell densities are the average of the two cultures and standard errors are indicated by the error bars or size of the data symbol. Samples were taken at day 6, 8, 22 and 43 for dry wt and analysis and are indicated by the arrows.

From an initial density of 3.5×10^2 cells ml^{-1} the culture increased by 3.5 times to 1.2×10^3 cells ml^{-1} at late logarithmic phase (day 6) which then increased 10-fold to 1.3×10^4 cells ml^{-1} at stationary phase (day 41) (Fig. 1). The microalgal biomass (dry weight per unit volume of culture) increased with increasing age of culture, from 8.5 mg l^{-1} at day 6 (late logarithmic phase) to 127 mg l^{-1} at day 43 (stationary phase). Although the growth rates and cell densities are low relative to those of other algal classes, this is not unusual for dinoflagellates, which only rarely exhibit high cell densities or growth rates exceeding one doubling per day (Taylor and Pollingher, 1987; Parker et al., 2002). Growth rates of one doubling every 3 days and cell densities of 10^3–10^4 are typical (e.g. Parker et al., 2002).

2.2. Lipid content and yield

The lipid content increased 2-fold, from logarithmic growth to linear growth phase (Table 1), then declined at stationary phase. The total fatty acid content as expected mirrored the trend in lipid content (Table 1) largely due to a decrease in polar lipids. In contrast, the sterol content did not reach a maximum at linear growth phase but continued to increase through to stationary phase (Table 1) which suggests, unlike the fatty acids they are not used as a energy source. There was also a large increase in the lipid yield (mass of lipid per volume of culture) from 1.3 mg l^{-1} at logarithmic to 30.1 mg l^{-1} at stationary phase which is due to the increase in biomass density (Table 1). Note that these values are about an order of magnitude less than has been achieved for some diatoms cultured for alternative fuels (McGinnis et al., 1997).

These results indicate that growth phase in batch cultures is an important factor which can influence the lipid content. Though many microalgae do not make large amounts of lipid, a rare few accumulate over 75% of their cell dry weight as neutral lipid (triacylglycerols), particularly as a result of environmental changes or stresses such as nitrogen limitation, salinity, or high temperature (Murphy, 2001). Phosphorus limitation has also been shown to lead to increased lipid content in some diatoms and haptophytes, but decreased lipid content have been observed in some green flagellates (Reitan et al., 1994).

Table 1
Content and yields[a] of major lipid constituents of *Gymnodinium* sp. CS-380/3 batch culture at different growth phases

	Late logarithmic phase		Linear phase	Stationary phase
	Day 6	Day 8	Day 22	Day 43
Culture densities at harvest (cell ml^{-1})[b]	1210±270	1815±45	4970±620	12 200±700
Algal biomass density (mg l^{-1})[c]	8.5±2.5	10.5±0.5	35.0±1.0	127±3
Lipid content (% dry wt)[d]	15.6±1.1	18.3±4.7	29.6±0.2	22.6±1.3
Lipid yield (mg l^{-1})	1.3±0.3	1.9±0.4	10.4±0.4	30.1±2.4
PL (% dry wt)[d]	13.2±0.5	15.0±0.2	21.8±0.2	15.0±0.2
PL (mg l^{-1})	1.1±0.3	1.6±0.3	7.7±0.3	20.0±1.6
TAG (% dry wt)[d]	1.2±0.3	1.8±0.1	5.8±0.1	6.5±0.4
TAG (mg l^{-1})	0.10±0.03	0.19±0.1	2.0±0.1	8.7±0.5
Total fatty acid content (% dry wt)	9.5±0.4	8.2±2.7	17.5±0.8	13.8±0.3
Total fatty acid yield (mg l^{-1})	0.9±0.3	0.9±0.3	6.1±0.5	17.6±0.9
Sterol content (% dry wt)	0.258±0.002	0.296±0.003	0.419±0.001	0.60±0.04
Sterol yield (mg l^{-1})	0.022±0.006	0.031±0.001	0.147±0.004	0.38±0.01
Sterol/fatty acid ratio[e]	0.03	0.04	0.02	0.04

[a] Data based on single analyses of duplicate cultures; PL = polar lipids, TAG = triacylglycerols.
[b] Cell density at start of experiment was 345±111 cell ml^{-1}.
[c] All masses in this table refer to dry weight of algae.
[d] Based on gravimetric determination.
[e] Ratios calculated using % dry wt.

2.3. Lipid classes

As the culture proceeded from logarithmic to linear to stationary phase the proportion of triacylglycerols (TAG) increased, and the polar lipid (PL) fraction decreased (Table 2). The high proportion of polar lipids (85% of total lipid at day 6) (mainly phospholipids and glycolipids) in *Gymnodinium* sp. is typical of most microalgae as these lipids help maintain the integrity of membranes of which phospholipids are the main components. Glycolipids are especially abundant in chloroplast membranes.

There was almost 4 times as much triacylglycerols at stationary phase than at logarithmic growth phase (Table 2). Triacylglycerols have also been found to be a major lipid class in other gymnodinoid species (*Symbiodinium microadriaticum*, and *Gymnodinium sanguineum* and other *Gymnodinium* species) that we have studied (unpublished results). Triacylglycerols function as storage lipids and thus amounts in most microalgae are usually lowest during logarithmic phase of growth (Volkman, 1989), but increase in stationary phase growth as nitrogen or phosphorus is depleted (Dunstan et al., 1993; Reitan et al., 1994; Brown et al., 1996). This effect is clearly seen here where the PL/TAG ratio changed from 11.8 at day 6 to 3.8 at linear phase to 2.3 at stationary phase. Triacylglycerol content can also be affected by other factors such as light-dark cycle and light intensity (Brown et al., 1996). This agrees with the suggestion by Gordillo et al. (1998) that the ratio of triacylglycerols to phospholipids can be used as an indicator of a physiological stress that uncouples C and N metabolism and affects the growth rate.

Table 2
Lipid class composition of *Gymnodinium* sp. CS-380/3 batch culture at different growth phases

Lipid class[a]	% of total lipid±S.E.[b]			
	Late logarithmic phase		Linear phase	Stationary phase
	Day 6	Day 8	Day 22	Day 43
Hydrocarbons[c]	1.7±0.5	1.5±0.3	1.3±0.2	1.2±0.1
Triacylglycerols	7.5±2.0	10.0±0.5	19.5±0.5	28.8±1.6
Free fatty acids	1.3±0.7	0.7±0.5	1.3±0.1	0.7±0.2
4-Methyl sterols	1.4±0.1	1.1±0.1	0.9±0.1	1.1±0.1
4-Desmethyl sterols	1.6±0.6	1.1±0.2	0.4±0.1	0.6±0.2
Unknown[d]	1.8±0.4	3.5±0.6	3.0±0.8	1.2±0.1
Polar lipids[e]	84.7±3.6	82.1±1.0	73.6±0.8	66.4±1.4
Total	100	100	100	100

[a] Sequence based on elution order.
[b] Based on analysis of duplicate cultures and two determinations for each culture ($n = 2$).
[c] May include sterol esters and wax esters if present.
[d] Includes pheophytin but unlikely to contain diacylglycerol or monoacylglycerols since these coelute with the sterols and polar lipids respectively using this solvent system.
[e] May include chlorophyll, polar carotenoids, phospholipids, lysophospholipids and glycolipids (based on unpublished analysis of *Scrippsiella* sp.).

2.4. Fatty acids

The main fatty acids in all growth phases were: 16:0, 18:5(n-3), 20:5(n-3) and 22:6(n-3) (Table 3 and Fig. 2). These fatty acids are typical of dinoflagellates (Nichols et al., 1984; Hallegraeff et al., 1991; Mansour et al.,

1999a,b) and some haptophytes (Holz, 1981, Nichols et al., 1987, Bell et al., 1997).

Minor amounts of a very long chain highly-unsaturated fatty acid, 28:8(n-3) were found. Recently 28:8(n-3) and 28:7(n-6) were identified for the first time in dinoflagellates cultured phototrophically (Mansour et al., 1999a) and in a dinoflagellate cultured heterotrophically (Van Pelt et al., 1999). These novel fatty acids appear to be unique to dinoflagellates and may therefore be useful as diagnostic chemical markers (e.g. Volkman et al., 1998).

There were substantial changes in the proportions of some individual fatty acids [16:0 and 18:5(n-3)], but not others [20:5(n-3) and 22:6(n-3)], with growth phase (Table 3). The largest increase (as a percent of total fatty acids) was shown by palmitic acid (16:0) and the largest decrease by 18:5(n-3). There was little change in the proportion of other minor fatty acids with changes in growth phase.

Other workers have reported that stationary phase cultures typically have a lower degree of unsaturation (Dunstan et al., 1993; Brown et al., 1996). In contrast Hallegraeff et al. (1991) found that stationary phase cultures of *G. catenatum* actually had slightly elevated proportions of 20:5(n-3) (+2%), 22:6(n-3) (+5%) and a slightly lower levels of 16:0 (−2%) compared with logarithmic phase cultures. Despite changes in the relative proportions of the main fatty acids, the fatty acid composition remained typical of dinoflagellates at all stages of growth, and therefore can still be used as a distinguishing chemotaxonomic characteristic of this algal class.

Table 3
Fatty acid composition (% of total fatty acids) of *Gymnodinium* sp. CS-380/3 batch culture at different growth phases

Fatty acid	Percentage composition			
	Late logarithmic phase		Linear phase	Stationary phase
	Day 6	Day 8	Day 22	Day 43
Saturates				
14:0	0.8±0.1	0.9±0.1	1.0±0.1	1.4±0.1
15:0	0.5±0.1	0.5±0.1	0.5±0.1	0.6±0.1
16:0	18.7±0.1	20.5±0.1	23.4±0.1	26.7±0.3
18:0	1.7±0.1	1.7±0.1	1.7±0.1	2.1±0.1
Sum	21.8±0.1	23.7±0.2	26.7±0.1	30.8±0.4
Monounsaturates				
18:1(n-9)	1.1±0.1	1.5±0.1	3.4±0.2	2.9±0.1
18:1(n-7)	0.3±0.1	0.2±0.1	0.0±0.1	0.7±0.1
Sum	1.4±0.1	1.7±0.1	3.4±0.2	3.6±0.3
Polyunsaturates				
18:2(n-6)	4.4±0.1	4.5±0.1	5.1±0.1	6.0±0.2
18:3(n-3)	–[a]	–	–	1.2±0.1
18:4(n-3)	2.6±0.1	3.1±0.1	3.8±0.1	1.8±0.1
18:5(n-3)	21.9±0.4	20.5±0.1	15.5±0.7	12.4±0.5
20:5(n-3)	12.3±0.2	11.4±0.2	9.9±0.4	8.0±0.2
22:6(n-3)	28.2±0.1	28.4±0.1	27.8±0.8	31.3±0.4
28:8(n-3)	2.2±0.1	1.9±0.1	1.8±0.1	1.3±0.1
Sum	71.6±0.8	69.8±0.3	63.8±0.3	62.1±0.8
Sum others	5.2±0.7	4.8±0.4	6.0±0.2	3.5±0.1
Sum total	100	100	100	100

[a] Not detected.

The content per unit dry weight of all the major fatty acids maximized during linear growth phase and then decreased at stationary phase. In particular, 22:6(n-3) and 16:0 showed the most pronounced effect and underwent a 2-fold increase from logarithmic to linear phase (Fig. 2) and remained above levels at logarithmic phase. In contrast, increase in 18:5(n-3) and 20:5(n-3) were less and these values returned to levels close to those of logarithmic phase (Fig. 2).

2.5. Sterols

Gymnodinium sp. CS-380/3 contains a complex mixture of sterols, most of which contain a methyl group at C-4. The only 4-desmethyl sterols were cholesterol, which was a minor component at all growth stages, and zymosterol (cholesta-8,24-dien-3β-ol) which appeared as a minor component (1.7%) at day 43 (stationary phase) (Table 4). The major sterol at days 6 and 8 (late logarithmic phase) was dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol), which is the major sterol in many dinoflagellates (e.g. Withers et al., 1979; Mansour et al., 1999b), together with only slightly smaller contents of its saturated analogue dinostanol. In linear and stationary phase cultures, the proportion of stenol to stanol was reversed (Table 4 and Fig. 3). Rather unexpectedly, two isomers of dinostanol were found and these were

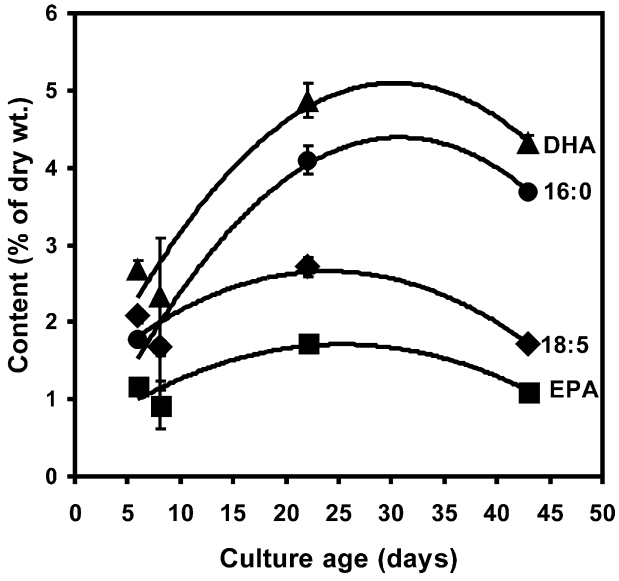

Fig. 2. Effect of growth phase on the content (% of dry wt of algae) of the main fatty acids in *Gymnodinium* sp. CS-380/3. Days 6–8 (late logarithmic phase), day 22 (linear phase) and day 43 (stationary phase).

Table 4
Sterol composition (% of total sterols) of *Gymnodinium* sp. CS-380/3 batch cultures at different growth phases

Sterol	Structure[d]	Percentage composition			
		Late logarithmic phase		Linear phase	Stationary phase
		Day 6	Day 8	Day 22	Day 43
C29:2 sterol $\Delta^{5,8(14)}$ 4,4-diMe?		0.60±0.05[a]	0.19±0.1	–[b]	–
Cholest-5-en-3β-ol (cholesterol)	1	1.04±0.29	1.16±0.1	0.69±0.04	0.46±0.15
Cholesta-8,24-dien-3β-ol (zymosterol)		–	–	–	1.73±0.24
C30:1 sterol		1.59±0.01	1.53±0.33	2.46±0.04	1.69±0.56
4α,24-Dimethyl-5α-cholest-22E-en-3β-ol	2	0.99±0.21	0.94±0.05	1.15±0.05	0.86±0.02
4α,24-Dimethyl-5α-cholestan-3β-ol	3	2.00±0.01	2.78±0.20	4.60±0.1	10.4±0.6
4α,23,24-Trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	4	40.9±0.8	39.3±0.7	33.3±0.4	28.7±0.2
4α,23,24-Trimethyl-5α-cholest-8(14)-en-3β-ol	5	13.1±0.1	11.0±0.6	11.1±0.1	7.50±0.35
Unknown[c]		5.23±0.21	5.73±0.32	5.71±0.09	3.82±0.71
4α,23S,24R-Trimethyl-5α-cholestan-3β-ol	6	1.77±0.02	2.02±0.17	3.28±0.14	7.60±1.04
4α,23R,24R-Trimethyl-5α-cholestan-3β-ol (dinostanol)	7	32.7±0.4	35.4±1.6	37.8±0.3	37.2±1.5
Sum		100.0	100.0	100.0	100.0

[a] Standard error of analysis of duplicate cultures.
[b] Not detected.
[c] Unknown sterol coelutes with dinosterol on an HP-5 Ultra 2 column under these chromatographic conditions, but is partially resolved on HP-1 capillary column.
[d] See Fig. 4 for side-chain structures of sterols.

identified as the 23*S*,24*R* and 23*R*,24*R* epimers by reference to retention data and mass spectra reported by Harvey et al. (1988) and Volkman et al. (1999). 4α,23,24-Trimethyl-5α-cholest-8(14)-en-3β-ol (which comprised 7.5–13.1% of the total sterols) is also comparatively rare in dinoflagellates, having only been reported in some species of *Scrippsiella* (Harvey et al., 1988) and a *Gymnodinium* sp. (Mansour et al. 1999b).

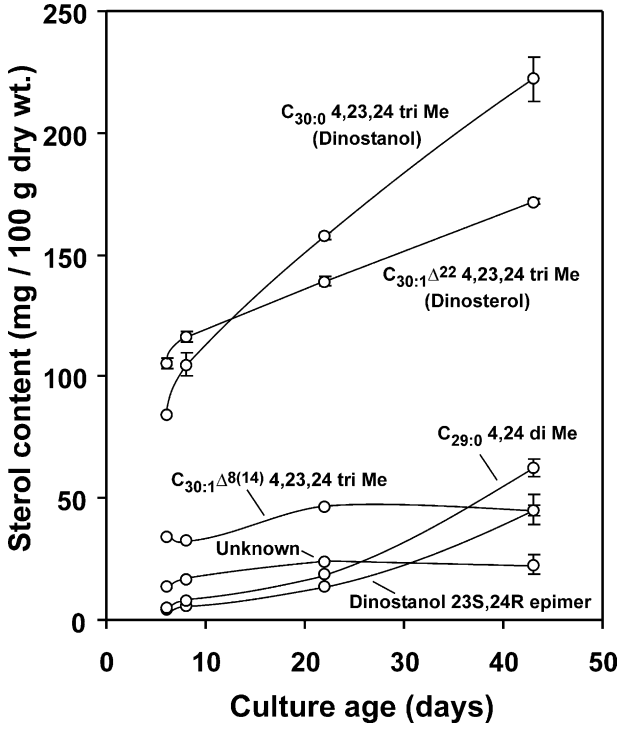

Fig. 3. Effect of growth phase on the amounts per dry wt of algae of the main sterols in *Gymnodinium* sp. CS-380/3. Days 6–8 (late logarithmic phase), day 22 (linear phase) and day 43 (stationary phase).

The pathways by which the side-chains of 4-methyl sterols in dinoflagellates are formed leading to the formation of dinosterol are now fairly well established (e.g. Withers et al., 1979; Giner, 1993). This sequence of side-chain alkylation and desaturation and double bond reduction accounts for the major sterols in *Gymnodinium* (Fig. 4). The role of some of the minor sterols, such as those with Δ^8-unsaturation, is less clear although they may result from transformation of lanosterol, which has been shown to be the key intermediate, rather than cycloartenol, in dinoflagellate sterol biosynthesis (Giner and Djerassi, 1991).

It has been suggested that the fully-saturated 4-methyl sterol dinostanol is formed by reduction of the Δ^{22} side-chain double bond in dinosterol (Volkman et al., 1984; Piretti et al., 1997), although evidence for a specific Δ^{22} saturase enzyme is lacking. Indeed, Ellouz and Lenfant (1969) showed that in a myxomycete, 24-ethylcholest-22-en-3β-ol is not a precursor of 24-ethylcholestanol indicating the lack of a specific saturase in that organism. Dinostanol is common in dinoflagellates (e.g. Alam et al., 1981, 1984), but only a single isomer is usually reported. Other species of *Gymnodinium* have been reported to contain a single dinostanol isomer (e.g. Mansour et al., 1999b), but two isomers have been reported in some species of *Scrippsiella* (Harvey et al., 1988) and *Prorocentrum* (Volkman et al., 1999).

The alkyl groups at C-24 in the sterols in most microalgae and higher plants usually show a single stereochemistry consistent with addition of hydrogen atoms from one face or the other, but not both. For

example, the reduction of 24-ethyldesmosterol to sitosterol in plants proceeds as an anti-addition of hydrogen atoms in a highly stereospecific reaction (Fujimoto et al., 1998). It is intriguing, then, that there is an increasing proportion of the "uncommon" dinostanol isomer having the opposite configuration at C-23 as the culture approaches stationary phase, even though the sum of dinosterol and the two dinostanol isomers is almost identical (75±2%) at all growth stages. If dinostanol is formed simply by reduction of dinosterol then our observations imply that the enzymatic reaction is not stereospecific. Alternatively, the dinostanol isomers might result from quenching of the intermediate formed from methionine addition to C-23 in 4α,24-dimethyl-5α-cholest-22E-en-3β-ol (Fig. 4), simultaneously leading to less dinosterol formation and the presence of two epimers. A third possibility is that dinostanol arises from reduction of 4α,23,24-trimethyl-5α-cholest-8(14)-en-3β-ol which is present in significant amounts (7.5–13.1%) at all growth phases (Table 4), although this appears unlikely since there is no evidence that the $\Delta^{8(14)}$ sterol exists as two epimers.

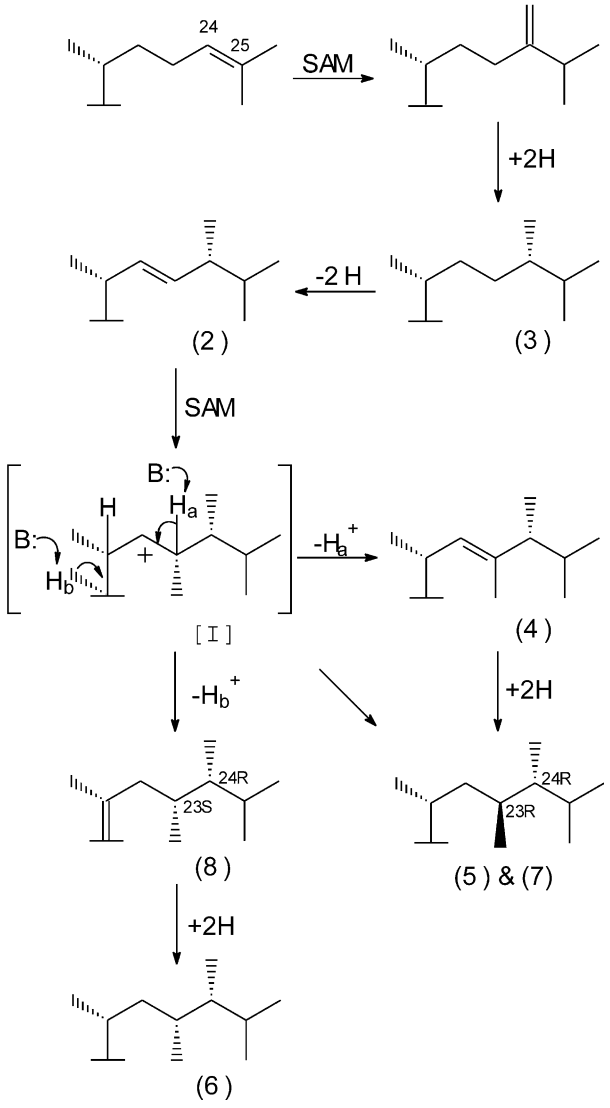

Fig. 4. Likely pathway, adapted from the work of Withers et al. (1979) and Giner (1993), of side-chain desaturation, saturation and alkylation via SAM (S-adenosyl-1-methionine) leading to the formation of the major sterols [3 = 4α,24-dimethyl-3alpha;-cholestan-3β-ol, 4 = 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol), 5 = 4α,23,24-trimethyl-5α-cholest-8(14)-en-3β-ol and 7 = 4α,23R,24R-trimethyl-3α-cholestan-3β-ol (dinostanol)] found in *Gymnodinium* sp. CS-380/3. Dinostanol (7) might arise from direct reduction of dinosterol (analogous to the sequence 2 to 3), in which case a single epimer with stereochemistry 23*R*,24*R* is expected, or perhaps from the cationic intermediate (I). Peridinosterol (8) can also be formed from this intermediate (Giner, 1993), but this sterol was not found in *Gymnodinium* sp. CS-380/3.

The same major sterols were present at all growth stages, despite small to moderate differences in their proportions (Table 4). The proportion of dinosterol continuously decreased as the culture aged, while stanols (i.e. sterols lacking a double bond in the ring system) all increased in relative abundance. When the content of the sterols per unit dry weight of alga was plotted a somewhat different picture emerged (Fig. 3). The production of each of the major sterols actually increased with age of culture, which mirrored the trend seen for total lipid and triacylglycerol content. The only exception was the content of the $C_{30:1}$ sterol 4α,23,24-trimethylcholesta-8(14)-en-3β-ol which remained static in linear and stationary phase (days 22 and 43). The changes in the slopes of the curves for individual sterols indicate changes in the production rates, but clearly there was no block to the production of any of the major sterols in stationary phase.

The sterol compositions have been considered to be more stable than lipid classes or fatty acid compositions with changing growth stage. Hence, they have been used as biochemical markers of microalgal-derived organic matter in marine sediments (Volkman, 1986), and chemotaxonomic markers for different species groupings within the Dinophyceae (Withers, 1987). However, a great diversity of sterol patterns can be found between some species of the same algal class, even between strains from the same genus (e.g. Mansour et al., 1999b; Volkman et al., 1999), so it is important to establish to what extent this reflects genetic differences or simply responses to different culturing conditions.

A few studies have reported large compositional changes in sterol distributions with growth phase in some microalgae (e.g. Ballantine et al., 1979; Piretti et al., 1997), such that particular sterols were found only at certain growth stages. However, the results of Alam et al. (1984) seem to be more typical. These authors examined the sterol composition of *Heterocapsa niei* on six occasions from 6 to 41 days after inoculation and showed that the same sterols were present at all growth stages. Cholesterol, dinostanol and 24-methylcholesterol contents showed little change, but the contents of the two major sterols 4,24-dimethylcholestanol and

dinosterol co-varied, with the latter increasing with time. This relationship is consistent with the idea that 4,24-dimethylcholestanol is desaturated and further alkylated to produce dinosterol as shown in Fig. 4.

Similarly, in our study, there were only small to moderate changes in the proportions of the sterols, and only one new sterol was identified in stationary phase. This is despite the fact that we tried to accentuate any changes by comparing cells in late logarithmic phase (days 6–8) with those that had been in stationary phase for 11 days (day 43). In this strain, sterol profiles are reasonably stable at different growth phases and over an extended culture age. We can thus be more confident in suggesting that the greatly divergent sterol profiles previously reported in species of this genus (Mansour et al., 1999b) are likely to be mainly due to genetic differences between strains.

3. Concluding remarks

While the proportions of the individual fatty acids and sterols may be affected by growth phase, the overall fatty acid profile is still characteristic of dinoflagellates and the sterol profile is essentially the same and hence is a useful chemotaxonomic feature of this strain. The maximized lipid (TAG, PL and DHA) content at linear growth phase suggests that growth phase may be manipulated to optimise these components. This could be useful for the production of DHA-lipid rich microalgae for aquaculture feeds/supplements, enrichment of live feeds or DHA-rich oil for human nutraceuticals.

4. Experimental

4.1. Culturing and harvesting

Gymnodinium sp. CS-380/3, a 35 μm diameter, unarmoured, non-chain forming member of the Gymnodinales is a common dinoflagellate in coastal south-east Australian waters. It was obtained from the CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/microalgae/collection.html), although originally isolated from coastal seawater from Devonport, Tasmania, Australia. Duplicate small-scale pre-experimental 200 ml cultures with 10% inoculum (20 ml) reached late-logarithmic phase after 7 days of growth (results not shown). These results were used as a basis for conducting a larger-scale experiment.

Duplicate 2 l experimental cultures were grown without aeration (due to the fragile nature of this alga) in two 3 l Erlenmeyer flasks, starting with a 175 ml inoculum from combined pre-experimental cultures made up to ~2 l with GSe medium [a modification of the GP medium of Loeblich (1975), see Blackburn et al., 2001). Initial cell density was 345 cell ml^{-1}. The cultures were maintained at 18.5 °C under 80 μmol photons PAR m^{-2} s^{-1} of cool white fluorescent light (measured with a Biospherical Optics light meter) on a 12:12 h L:D cycle.

Cultures were gently mixed to ensure a homogenous sample prior to sampling for cell counts, dry weights and harvesting. Cell density was estimated from 2 ml samples using a Sedgwick–Rafter slide (Guillard, 1973), each day until day 8, then once every 3–4 days until the end of the experiment. The specific growth rate (μ) in d^{-1} was calculated according to the equation $\mu = \ln(N_2/N_1)/(t_2-t_1)$ where N is cell densities and t is the time (Guillard, 1973). For chemical analysis, 400 ml of culture were removed at the same time during the photoperiod on days 6, 8 (late logarithmic phase), 22 (linear growth phase) and 43 (stationary phase) and the cells harvested by centrifugation at 3000–4500 g for 10 min. Dry weights were determined by filtering 100 ml aliquots (in duplicate) of culture at harvest through ashed and pre-weighed glass-fibre filters (Whatman GF/F 47 mm diameter). Residual salt was removed by rinsing the filter with 20 ml of 2 M ammonium formate and oven drying for 2 h at 105 °C. The filter with sample was reweighed to determine the dry weight of biomass.

4.2. Extraction

Cultures were centrifuged and the microalgal pellet extracted in $CHCl_3$–MeOH–H_2O (1:2:0.8, v/v/v) by a modified version of Bligh and Dyer's (1959) method, using successive ultrasonication (15 min) and centrifugation (5–6 times). To initiate phase separation, chloroform and purified water (Milli-Q system, Millipore) were added to the combined extracts to give a final $CHCl_3$–MeOH–H_2O ratio of 1:1:0.9 (v/v/v). The chloroform layer was rotary-evaporated and lipid content determined gravimetrically. The extracts were then reconstituted in chloroform (1.8 ml) and stored under nitrogen at −20 °C until analysis.

4.3. Lipid class analysis

Lipid classes were analysed by Iatroscan TLC-FID (Mark V) with Chromarod S-III silica rods, using the method of Volkman and Nichols (1991).

4.4. Fatty acid and sterol derivatization

Fatty acid methyl esters (FAME) were formed by transesterification of the total lipid by heating with MeOH–HCl–$CHCl_3$ (10:1:1, v/v/v) at 80 °C for 2 h. The FAME and sterols were extracted into hexane-$CHCl_3$ (4:1, v/v) and stored under nitrogen at −20 °C. Immediately before GC and GC–MS analysis, the transmethylated lipid extract was treated with *bis*

(trimethylsilyl)trifluoroacetamide (BSTFA) for 30 min at 80 °C to derivatize sterols to their trimethylsilyl ethers.

4.5. Capillary gas–liquid chromatography (GC)

FAME and sterol trimethylsilyl ethers were analysed with a Varian High Temperature Series 5410 gas chromatograph with a Series 8100 autosampler, a septum-equipped programmable injector (SPI) and a flame-ionization detector. Samples were injected at 45 °C onto a non-polar methyl-silicone fused-silica capillary column (HP-1, 50 m×0.32 mm i.d., Hewlett Packard). After 1 min, the oven temperature was raised to 120 °C at 30 °C min^{-1} and then to 310 °C at 4 °C min^{-1}. This final temperature was maintained for 10 min. The initial temperature of the SPI was 50 °C, which was held for 0.15 min, then raised to 320 °C at a rate of 150 °C min^{-1} and maintained for 5 min. Hydrogen was used as the carrier gas and the detector set at 310 °C.

FAME were quantified by adding *n*-tricosanoic acid methyl ester (23:0 FAME) in chloroform as an internal standard. Sterol-TMS ethers were quantified with *n*-docosane (*n*-C_{22}) as an internal standard and identified by comparing published relative retention times and mass spectral data (e.g. Hallegraeff et al., 1991; Volkman et al., 1999).

4.6. Gas chromatography–mass spectrometry (GC–MS)

Electron impact (EI) GC–MS was carried out on a Fisons MD-800 with an on-column injector at 45 °C. The sample was injected into a retention gap attached to an HP-5 Ultra 2 bonded-phase column (50 m; 0.32 mm i.d.; 0.17 μm film thickness). The initial temperature of 45 °C was held for 1 min, followed by temperature programming at 30 °C min^{-1} to 140 °C then at 3 °C min^{-1} to 310 °C where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; transfer line 310 °C; source temperature 250 °C; scan rate 0.8 scans s^{-1} and mass range 40–650 Dalton. Mass spectra were acquired and processed with Fisons Masslab software.

4.7. Statistical treatment of data

ANOVA: Single Factor ($\alpha = 0.05$) was applied using the statistical options in Microsoft Excel 97 SR-2.

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