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Cryptophyceae and rhodophyceae; chemotaxonomy, phylogeny, and application

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Abstract

The biochemical compositions of seven strains of marine cryptomonad and a rhodophyte were determined in logarithmic phase batch (1.4 L flask) and semi-continuous (10 L carboy) culture. Lipid ranged from 13% to 28%, protein ranged from 53% to 68%, and carbohydrate ranged from 9% to 24% of the organic weight. The major lipid classes in the species examined were polar lipids (78–88% of total lipid). The major sterol in the Cryptophyceae and the Rhodophyceae was 24-methylcholesta-5,22E-dien-3β-ol (62–99% of total sterols); which is also the major sterol in some diatoms and haptophytes. Smaller proportions of cholest-5-en-3β-ol (1–17.7%) were also found in the Cryptophyceae. Most cryptomonads contained high proportions of the $n-3$ polyunsaturated fatty acids (PUFA), 18:3$n-3$ (20.7–29.9% of the total fatty acids), 18:4$n-3$ (12.5–30.2%), 20:5$n-3$ (7.6–13.2%) and 22:6$n-3$ (6.4–10.8%). However, the blue–green cryptomonad *Chroomonas placoidea* was characterized by a low proportion of 22:6$n-3$ (0.2% of total fatty acids), and a significant proportion of 22:5$n-6$ (4.5%), and the presence of 24-ethylcholesta-5,22E-dien-3β-ol (35.5% of total sterols). The fatty acid composition of the rhodophyte *Rhodosorus* sp. was similar to those of the Cryptophyceae except for lower proportions of 18:4$n-3$ and lack of C_{21} and C_{22} PUFA. It is postulated that the primary endosymbiosis of a photosynthetic $n-3$ C_{18} PUFA-producing prokaryote and a eukaryotic host capable of chain elongation and desaturation of exogenous PUFA, resulted in the Rhodophyceae capable of producing $n-3$ C_{20} PUFA. The secondary endosymbiosis of a photosynthetic $n-3$ C_{20} PUFA-producing eukaryote (such as a *Rhodosorus* sp. like-rhodophyte) and a eukaryotic host capable of further chain elongation and desaturation, resulted in the Cryptophyceae being capable of producing $n-3$ C_{20} and C_{22} PUFA de novo. Selected isolates were examined further in feeding trials with juvenile Pacific oysters (*Crassostrea gigas*). *Rhodomonas salina* CS-24(containing elevated 22:6$n-3$) produced high growth rates in oysters; equivalent to the microalga commonly used in aquaculture, *Isochrysis* sp. (T.ISO).

Keywords: Cryptophyceae; Rhodophyceae; Chemotaxonomy; Phylogeny; Aquaculture; Amino acids; Polyunsaturated fatty acids; Sterols

1. Introduction

Most members of the Rhodophyceae are marine macroalgae, however species such as *Porphyridium*, *Rhodosorus*, and *Rhodella* are unicellar coccoid microalgae. Ultrastructural (cellular membranes), genetic and chemical evidence suggests that extant eukaryotic algae from classes such as the Rhodophyceae and Chlorophyceae originated from the incorporation of a prokaryotic photosynthetic unicellular cyanobacterium into a non-photosynthetic eukaryotic protist host in an endosymbiotic association (Kenyon and Stanier, 1970; Lee, 1972; Whatley, 1981; Cavalier-Smith, 2000; Nozaki et al., 2003). Over time and with significant modifications, the prokaryotic symbionts from this primary endosymbiotic event became functional chloroplasts (Lee, 1972; Whatley, 1981; Cavalier-Smith, 2000). The result was a photosynthetic eukaryotic unicell

Abbreviations: n, nucleus and p, plastid of rhodophyte; n′, nucleomorph; N, nucleus and P, plastid of cryptophyte; C_{18}, C_{20} and C_{22} represent production of 18, 20, 22 carbon chain length $n-3$ polyunsaturated fatty acids (PUFA) respectively; flagella of stylised cryptophyte and other organelles not shown.

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containing the phycobiliproteins, carotenoids and chlorophyll *a* from the endosymbiotic cyanobacterium as occurs in the Rhodophyceae.

Members of the Cryptophyceae are marine or freshwater flagellated unicells and most are photosynthetic (Gillott, 1989). The chloroplast of algal groups such as the Cryptophyceae is believed to be derived from a photosynthetic eukaryotic endosymbiont in a secondary endosymbiotic event (Gillott and Gibbs, 1980; Douglas et al., 1991; Cavalier-Smith, 2000). Based on similar ultrastructural (cellular membranes, presence/absence of the nucleomorph), genetic (DNA and RNA) and chemical (storage carbohydrates, pigments and lipids) evidence as for the primary endosymbiosis, this photosynthetic eukaryotic endosymbiont was probably a primitive rhodophyte (Gillott and Gibbs, 1980), possibly similar to today's unicellular rhodophytes (Gillott, 1989; Cavalier-Smith et al., 1996). Presumed vestigial endosymbiont nuclei known as nucleomorphs have been identified in examples from the cryptomonads and dinoflagellates (Whatley, 1981; Kies and Kremer, 1989). Lee (1972) suggested that the host cell in such a scheme would likely be a colourless ancestral cryptomonad-like organism, Gillott and Gibbs (1980) suggests that it was a colourless flagellate and Cavalier-Smith (2000) further elaborates that it may have been a biciliate host. Prior to the endosymbiotic theory being posed, many authors previously had explained the similarities and differences between the fatty acid compositions of the various algal groups in relation to the then current thinking of phylogenetic relationships (e.g. Nichols, 1970; Erwin, 1973). Since then, the causative relationship between the endosymbiosis theory of microalgal evolution and fatty acid composition has been suggested for chlorophyceaens, eustigmatophytes, diatoms and dinoflagellates (Sargent et al., 1995). The current study discusses this relationship with respect to the Rhodophyceae and Cryptophyceae.

The 18:2$n-6$ and 18:3$n-3$ PUFA (polyunsaturated fatty acids) are formed de novo by microalgae by successive (methylene interrupted) desaturation between the Δ9 bond and the methyl end of 18:1$n-9$. Subsequent Δ6 desaturation between the Δ9 bond and the carboxyl end of 18:3$n-3$ forms 18:4$n-3$. The longer chain C_{20} and C_{22} $n-3$ PUFA usually result from chain elongation and desaturation of these C_{18} PUFA. Evidence suggests that multicellular animals cannot synthesise both $n-3$ and $n-6$ polyunsaturated fatty acids de novo, however many species require them for normal development and growth (Sargent et al., 1995; Pereira et al., 2003). If provided with dietary C_{18} $n-3$ and $n-6$ PUFA, animals can chain-elongate and desaturate these to form the C_{20} and C_{22} $n-3$ and $n-6$ PUFA, with varying degrees of efficiency. In marine animals the production of these long-chain PUFA is generally relatively inefficient (Sargent et al., 1995), and because of this, marine food webs rely on the de novo synthesis and accumulation of C_{20} and C_{22} $n-3$ and $n-6$ PUFA primarily derived from algal sources. Therefore, as most marine animals are nutritionally dependant on dietary sources of these long-chain PUFA, photosynthetic microalgae are the foundation of most marine food webs. Similarly, the high proportions of the essential $n-3$ C_{20} and C_{22} PUFA in microalgae make them important feeds in mariculture operations to improve animal growth and survival.

In the present study, the biochemical compositions of seven strains of marine cryptomonad (Cryptophyceae) and one strain of red microalga (Rhodophyceae) were compared. The phylogenetic relationships of the Cryptophyceae and Rhodophyceae with respect to fatty acid biosynthesis and the endosymbiosis theory are considered. The nutritional value of three of the cryptomonads was examined in feeding trials with juvenile Pacific oysters (*Crassostrea gigas*).

2. Results and discussion

2.1. Proximate composition

Protein was the major organic constituent of all strains grown to logarithmic phase in batch culture (Table 1). Protein ranged from 53% (*Rhodomonas salina* CS-174) to 68% (*Rhodosorus* sp. CS-210) of the organic weight (OW) (Table 1). Cryptomonads typically contain higher amounts of protein than other microalgae (McCausland et al., 1999; Renaud et al., 1999). Of all the strains examined carbohydrate ranged from 9% (*R. salina*. CS-24) to 24% (*R. salina* CS-174) of the OW, whereas lipid ranged from 13% (*Rhodosorus* sp. CS-210) to 28% (*R. salina* CS-24). The gross composition of the two cultures of *R. salina* (CS-174), were compared to assess variability between duplicate cultures and suggests that duplicate cultures for all strains were not necessary. Their compositions were almost identical, with 53–57% of their OW as protein, 21–24% carbohydrate and 22% lipid. Similarly the two different strains of *R. salina* (CS-174 and CS-24) also had very similar compositions (Table 1).

The major lipid classes in all strains of cryptomonad in logarithmic phase batch culture were the polar lipids, ranging from 78% to 87% of the total lipid (Table 1). Triacylglycerols were not detected (<0.3 pg/cell) in most species, except for the blue–green cryptomonad *Chroomonas placoidea* (12.9%), while low levels (<10%) of free fatty acids, sterols, hydrocarbons and/or wax esters and an unidentified pigmented lipid fraction were also detected. The duplicate batch cultures of *R. salina* CS-174 were similar with respect to lipid class (Table 1), fatty acid (Table 2) and sterol (Table 3) composition, as were the two different strains of *R. salina* (CS-174 and CS-24).

The biochemical composition of the microalgae grown under semi-continuous logarithmic phase culture used in this trial were also analysed. Protein concentrations were higher in *R. salina* CS-24 and *C. placoidea* ($56 \pm 4\%$ and $57 \pm 4\%$ of OW, respectively) than *R. maculata* and *Isochrysis* sp. (T.ISO) ($50 \pm 4\%$ and $46 \pm 5\%$). Carbohydrate levels were not significantly different in *Isochrysis* sp. (T.ISO),

Table 1
Species of cryptophyceae and rhodophyceae examined, growth temperatures, cell densities, proximate composition and lipid class composition from logarithmic phase batch cultures

	Cryptophyceae								Rhodophyceae
Species	*Rhodomonas* sp.	*Rhodomonas*/ *Proteomonas* sp.	*Proteomonas sulcata*	*Rhodomonas salina* (Wislouch) Hill et Wetherbee		*Rhodomonas salina* (Wislouch) Hill et Wetherbee	*Rhodomonas maculata* Butcher ex Hill et Wetherbee	*Chroomonas placoidea* Butcher	*Rhodosorus* sp.
CSIRO culture collection code:	CS-215	CS-694	CS-412	CS-174		CS-24	CS-85	CS-200	CS-210
				Replicate A	Replicate B				
Culture temperature (°C)	20	15	25	20	20	20	20	15	25
Cell density ($\times 10^5$ cells/ml)	3.1	5.1	12.5	3.6	3.7	3.6	2.7	3.3	[a]
Gross-composition % OW									
Lipid	18	22	18	22	22	28	23	25	13
Carbohydrate	19	15	19	24	21	9	20	11	19
Protein	62	63	63	53	57	63	57	64	68
Lipid classes (% of total lipid)									
Polar lipids	88	87	86	85	84	85	86	78	n.d.[b]
Pigments	2.7	1.5	2.6	2.8	3.2	3.5	5.1	1.4	n.d.
Sterols	2.3	4.6	3.6	2.3	2.2	1.2	1.7	1.7	n.d.
Free fatty acids	5.0	3.7	4.7	7.0	7.3	9.2	5.4	3.6	n.d.
Triacylglycerols	–[c]	–	–	–	–	–	–	13.0	n.d.
Hydrocarbons/wax esters	1.7	2.9	3.0	2.6	3.6	1.5	2.2	2.1	n.d.

[a] Cells clumped and could not be counted.
[b] Not determined as saponification was required to extract the lipids.
[c] Not detected.

Table 2
Relative concentrations of fatty acids (% of total fatty acids) and total cellular fatty acid content (pg/cell) of the cryptomonads and the rhodophyte from logarithmic batch and semi-continous culture

	Cryptophyceae											Rhodophyceae
	Rhodomonas sp.	*Rhodomonas*/ *Proteomonas* sp.	*Proteomonas sulcata*	*Rhodomonas salina* (Wislouch) Hill et Wetherbee		*Rhodomonas salina* (Wislouch) Hill et Wetherbee		*Rhodomonas maculata* Butcher ex Hill et Wetherbee		*Chroomonas placoidea* Butcher		*Rhodosorus sp.*
	CS-215	CS-694	CS-412	CS-174		CS-24		CS-85		CS-200		CS-210
Culture type:	Batch	Batch	Batch	Batch Replicate A	Batch Replicate B	Batch	Semi-continuous	Batch	Semi-continuous	Batch	Semi-continuous	Batch
Saturated fatty acids												
14:0	6.7	2.1	3.1	7.3	7.4	3.8	4.5	7.3	11.2	1.0	1.3	0.3
16:0	8.2	11.4	11.6	8.3	8.0	8.6	10.8	10.1	10.0	16.2	13.9	26.9
18:0	0.7	1.2	0.4	0.6	0.5	1.0	0.9	0.7	1.1	1.4	1.3	1.1
Subtotal[a]	15.8	14.9	15.2	16.4	16.1	13.5	16.1	18.4	22.3	18.7	16.5	28.3
Monoenoic fatty acids												
16:1$n-9$	0.5	0.7	0.7	0.5	0.4	0.2	0.2	0.3	1.5	0.4	1.2	0.1
16:1$n-13$t	2.4	2.1	1.7	1.9	2.0	2.1	[b]	2.0	[b]	1.8	[b]	0.9
16:1$n-7$	0.9	0.9	1.4	1.0	1.0	0.5	2.5	0.7	5.6	1.8	4.0	1.2
18:1$n-9$	0.6	0.9	1.0	0.7	0.6	0.5	2.2	0.9	1.3	2.8	2.8	9.3
18:1$n-7$	6.0	1.7	1.3	2.6	2.7	6.5	4.4	4.4	8.6	6.7	5.6	0.6
Subtotal	10.8	6.8	6.2	6.8	6.7	10.3	14.2	8.4	18.0	13.6	17.3	12.4
Polyenoic fatty acids												
16:2$n-4$	0.2	0.1	0.2	0.1	0.1	0.1	0.8	0.2	2.0	0.1	1.3	0.1
18:2$n-6$	5.3	0.5	2.9	14.7	14.0	1.4	0.6	2.1	1.8	9.3	9.5	5.7
18:3$n-6$	0.6	0.2	0.5	2.3	2.4	0.3	0.2	0.2	0.5	0.4	0.5	0.5
18:3$n-3$	29.9	22.0	29.3	21.3	21.6	20.7	19.4	26.6	29.9	27.2	19.4	26.5
18:4$n-3$	20.9	30.0	20.6	18.4	19.2	30.2	22.7	27.5	10.7	12.5	15.2	0.8
20:2$n-6$	tr[c]	0.1	tr	0.1	0.1	tr	–	0.1	0.7	0.1	0.3	0.1
20:3$n-6$	–[d]	tr	tr	0.1	0.1	–	–	–	–	tr	–	0.1
20:4$n-6$	0.6	0.2	0.6	2.4	2.6	0.2	–	0.2	–	0.3	0.3	3.7
20:3$n-3$	tr	0.1	0.1	tr	tr	0.1	0.2	0.1	0.3	0.1	–	0.5
20:4$n-3$	0.2	0.4	0.4	0.5	0.5	0.5	1.0	0.3	0.2	0.4	0.3	0.4
20:5$n-3$	7.6	12.3	12.6	9.3	9.1	13.2	12.2	7.7	5.4	11.0	12.4	20.7
22:5$n-6$	0.1	0.6	2.4	0.3	0.3	0.1	0.2	0.4	0.0	4.5	4.8	–
22:5$n-3$	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.2	–	tr	–
22:6$n-3$	6.7	10.8	7.8	6.5	6.4	7.8	7.1	6.4	4.1	0.2	0.4	–
Subtotal	72.2	77.8	77.6	76.1	76.6	74.9	66.4	71.9	56.7	66.4	64.7	58.9
fas pg/cell	8.7	5.1	2.2	6.5	6.0	7.4	nd[e]	11.2	nd	4.7	nd	nd
Others[a]	1.2	0.4	0.9	0.6	0.6	1.3	3.2	1.2	3.1	1.3	1.5	0.3

[a] Subtotals and "others" include minor contributions (<0.5% each) from i15:0, a15:0, i16:0, a17:0, i18:1, 15:0, 15:1$n-6$, 16:1$n-5$, 18:1$n-5$, 20:1$n-11$, 20:1$n-7$, 20:1$n-9$, 16:2$n-7$, 16:3$n-6$, 22:1$n-9$, 24:1$n-9$, 21:5$n-3$.
[b] 16:1$n-13$t could not be distinguished from 16:1$n-7$c in these samples.
[c] Trace <0.5% TFA.
[d] Not detected.
[e] Not determined.

Table 3
Relative concentrations of sterols (% of total sterols) and total cellular sterol content (pg/cell) of the cryptomonads and the rhodophyte from logarithmic batch culture

Sterol (% of total sterols)	Cryptophyceae								Rhodophyceae
	Rhodomonas sp.	*Rhodomonas*/ *Proteomonas* sp.	*Proteomonas sulcata*	*Rhodomonas salina* (Wislouch) Hill et Wetherbee		*Rhodomonas salina* (Wislouch) Hill et Wetherbee	*Rhodomonas maculata* Butcher ex Hill et Wetherbee	*Chroomonas placoidea* Butcher	*Rhodosorus* sp.
	CS-215	CS-694	CS-412	CS-174		CS-24	CS-85	CS-200	CS-210
				Replicate A	Replicate B				
Δ^7 *sterols*									
4,24-Dimethyl-5α-cholest-7-en-3β-ol	–[a]	–	–	–	–	–	–	–	5.8
4-Methyl-5α-cholesta-7,22-dien-3βol	–	–	–	–	–	–	–	–	12.3
24-Methyl-5α-cholest-7-en-3β-ol	–	–	–	–	–	–	–	–	3.4
24-Methyl-5α-cholesta-7,22E-dien-3β-ol	–	–	–	–	–	–	–	–	2.2
$\Delta^{5,7}$ *sterol*									
24-Methylcholesta-5,7,22-trien-3β-ol	–	7.5	–	–	–	–	–	–	–
Δ^5 *sterols*									
24-Methylcholest-5-en-3β-ol	–	–	–	–	–	–	–	–	2.5
24-Methylcholesta-5,22E-dien-3β-ol	98.7	91.5	97.3	98.9	99	98.1	82.3	62.5	71.8
24-Ethylcholesta-5,22E-dien-3β-ol	–	–	–	–	–	–	–	35.5	–
Cholest-5-en-3β-ol	1.3	1.0	2.7	1.1	1.0	1.9	17.7	2.0	–
Unidentified sterol	–	–	–	–	–	–	–	–	2.0
Total sterols pg/cell	0.16	0.13	0.05	0.20	0.20	0.14	0.23	0.14	n.d.[b]

[a] Not detected.
[b] Not determined.

R. maculata and *R. salina* CS-24 ($22 \pm 43\%$, $22 \pm 5\%$, $18 \pm 5\%$, respectively) but they all contained more than *C. placoidea* ($15 \pm 3\%$). Lipid levels did not differ between the species (range of $26 \pm 3\%$ to $30 \pm 3\%$). For these strains, OW constituted between 74% and 84% of the total DW. The protein concentrations of the cryptomonads cultured semi-continuously were similar to values in the same species from log phase batch cultures (Table 1), though the cultures were generally richer in carbohydrate and lipid.

2.2. Fatty acid composition and chemotaxonomy

The main saturated fatty acids in the cryptomonads grown in batch culture were 14:0 (1.0–7.4% of total fatty acids) and 16:0 (8.0–16.2%), however with the exception of 18:1n-7 (1.3–8.6%), individual monoenoic fatty acids were not abundant in the Cryptomonads (Table 2). Most strains contained high proportions of $n-3$ polyunsaturated fatty acids, 18:3$n-3$ (20.7–29.9%), 18:4$n-3$ (12.5–30.2%), 20:5$n-3$ (7.6–13.2% of total fatty acids) and 22:6$n-3$ (6.4–10.8%). The exception was *C. placoidea*; it had similar proportions of C_{18} and C_{20} $n-3$ PUFA as the other cryptomonads but the C_{22} $n-3$ PUFA were less abundant (22:6$n-3$ comprised only 0.2% of total fatty acids, and 22:5$n-3$ was not detected). In contrast, 22:5$n-6$ was a significant component (4.5%) of this species' lipids (Table 2). The three cryptomonads grown in semi-continuous culture had similar fatty acid compositions to when grown in batch culture (Table 2). Generally, there were very low levels of C_{16} and $n-6$ C_{20} or $n-6$ C_{22} PUFA detected in all strains examined (Table 2).

The fatty acid composition of the rhodophyte (*Rhodosorus* sp.), was similar to those of the Cryptophyceae (with high proportions of $n-3$ C_{20} PUFA), except that little 18:4$n-3$ and no C_{21} or C_{22} were detected. The lack of PUFA with chain-lengths greater than C_{20} in *Rhodosorus* sp. has also been reported for other unicellular rhodophytes (e.g. *Porphyridium* spp.; Cohen, 1990). Other coccoid non-motile eukaryotic microalgae such as the Eustigmatophyceae *Nannochloropsis* spp. and *Monodus subterraneus* also do not produce C_{22} PUFA, C_{20} being the longest PUFA produced (Volkman et al., 1993; Khozin-Golderg et al., 2002), while non-motile Chlorophyceae do not produce either C_{20} or C_{22} PUFA (Dunstan et al., 1992). C_{22} PUFA are also only minor components in other non-flagellated species such as the diatoms (Volkman et al., 1989; Dunstan et al., 1994) and benthic macroalgae (including macroalgal rhodophytes, – Jamieson and Reid, 1972). Some reasons for these reduced levels or absence of C_{22} PUFA in many non-motile species (such as the rhodophyte *Rhodosorus* sp.) are that they do not require it for normal cellular functions and therefore may be due to the absence (having never acquired the genes responsible for the elongase enzyme system) or loss of the gene responsible for synthesis of the elongase (due to lack of a functional use), or less likely very rapid β-oxidation. In contrast C_{22} PUFA are significant components in flagellated species such as cryptomonads (Table 2). The cryptomonads examined had PUFA distributions similar to other Cryptophyceae (Beach et al., 1970; Renaud et al., 1999), as well as the flagellated Pavlovales (Prymnesiophyceae, Volkman et al., 1989, 1991) and Dinophyceae (Mansour et al., 1999) with high proportions of C_{18}, C_{20} and C_{22} $n-3$ PUFA, but very low proportions of C_{16} PUFA. This implies that 22:6$n-3$ may be an important fatty acid involved in flagellate motility. It has been suggested that phospholids containing 22:6$n-3$ are important co-factors for fast kinetics receptor-activated systems typical of excitory and contractile cells, explaining the often high levels of this PUFA observed in flagellated cells (Infante and Huszagh, 1998). However, while many flagellated Prasinophyceae also contain the long-chain C_{22} PUFA, flagellated species such as *Tetraselmis* spp. (Volkman et al., 1989; Dunstan et al., 1992), and the flagellated Chlorophyceaen *Dunaliella tertiolecta* (Volkman et al., 1989) do not, indicating C_{22} are not an absolute requirement for flagella function.

The composition of the blue–green cryptomonad *C. placoidea* (Table 2) was different from other cryptomonads examined to date (Beach et al., 1970; Renaud et al., 1999), as it had negligible levels of 22:6$n-3$ but elevated 22:5$n-6$ instead. It is unlikely that the low level of 22:6$n-3$ was due to α-oxidation, as the 21:5$n-3$ was not significantly elevated relative to the other species (Table 2). Nor was it due to decarboxylation, as the level of the hydrocarbon n-$C_{21:6}$ was not elevated in this strain (data not presented). Pentaenoic C_{22} PUFA have also been identified as significant components [at the expense of 22:6$n-3$] in a species of *Pyramimonas* (Prasinophyceae; Dunstan et al., 1992), many marine macroalgae (Jamieson and Reid, 1972), and in *Pseudostaurosia pinnata* (formerly *Fragilaria pinnata*, Bacillariophyceae; Dunstan et al., 1994), but in all of these cases it was the $n-3$ isomer which was elevated, not the $n-6$ isomer as in *C. placoidea* (Table 2). *C. placoidea* was the only "blue" (main phycobiliprotein pigment being phycocyanin) cryptomonad examined, where as the others were "red' cryptomonads (main phycobiliprotein pigment being phycoerythrin). Both types were thought to have diverged from a similar ancestral stock with loss of either pigment (Cavalier-Smith et al., 1996), however it would seem that *C. placoidea* also has a reduced ability to produce $n-3$ C_{22} PUFA.

2.3. Sterol composition and chemotaxonomy

The sterol distributions in the microalgae examined here were characterised by a remarkably simple distribution, dominated in most cases, by the single C_{28} sterol 24-methylcholesta-5,22E-dien-3β-ol (62.5–99% of the total sterols, Table 3). This is consistent with the few previous reports of sterols in microalgae from the Cryptophyceae (Goad et al., 1983; Gladu et al., 1990). This sterol is also the major constituent in many diatoms (Volkman, 1986) and it shares the same 24S (24α) stereochemistry at C-24 (Goad et al., 1983). The presence of the corresponding C_{29} $\Delta^{5,22}$ sterol 24-ethylcholesta-5,22E-dien-3β-ol in *C. placoidea* (Table

3) indicates that this alga is able to further alkylate the C-24 position whereas the *Rhodomonas* spp. examined apparently lacked this ability.

A very different composition was reported for the sterols in another member of the Cryptophyceae, *Chilomonas paramoecium* where the main constituents were 24-methylcholesta-5,7,22-trien-3β-ol (ergosterol – also a minor sterol in *Rhodomonas/Proteomonas* sp. CS-694; 7.5%, Table 3), and 24-ethylcholesta-5,22-dien-3β-ol (present in *C. placoidea*; 35.5%, Table 3) (Patterson, 1991).

Rhodosorus sp. (Rhodophyceae) contained a suite of sterols not detected in the cryptophytes, including 24-methyl-5α-cholesta-7,22E-dien-3β-ol (2.2%), 24-methylcholest-5-en-3β-ol (2.5%), 24-methyl-5α-cholest-7-en-3β-ol (3.4%), 4,24-dimethylcholest-7-en-3β-ol (5.8%) and two unidentified sterols whose mass spectra were too weak for identification (Table 3). Another 4-methyl sterol – tentatively identified as 4-methyl-5α-cholesta-7,22-dien-3β-ol (although the double bond might be at positions 8(9) or 9(11)) – was also found in this alga (12.3%; Table 3). Note that 4-methyl sterols are not common in microalgae and high amounts are more usually associated with dinoflagellates (e.g., Volkman, 1986; Withers, 1987), and some haptophytes from the genus *Pavlova* (Volkman et al., 1990; Gladu et al., 1991).

A number of sterol analyses of *Porphyridium* species (Rhodophyceae) have been reported and these are very different from that of *Rhodosorus*. *Porphyridium* species typically have a sterol profile dominated by cholesta-5,22-dien-3β-ol with smaller amounts of cholest-5-en-3β-ol, cholesta-5,24-dien-3β-ol and 24-methylcholesta-5,7,22-trien-3β-ol (Teshima and Kanazawa, 1973; Beastall et al., 1971, 1974), none of which were detected in *Rhodosorus* sp. (Table 3). However, *Porphyridium* species do contain small proportions of 24-methylcholesta-5,22-dien-3β-ol, (Beastall et al., 1971, 1974), the major sterol of all species in our present study. 4-Methyl sterols were found in marine *P. cruentum* (26% of total sterols) and the freshwater *P. aerugineum* (6% of total sterols); both contain 4-methyl-5α-cholest-8-en-3β-ol and 4,24-dimethyl-5α-cholest-8-en-3β-ol, while *P. cruentum* also contained 4-methyl-5α-cholesta-8,22-dien-3β-ol and 4,24-dimethyl-5α-cholesta-8,22-dien-3β-ol (Beastall et al., 1974). Both of these species of *Porphyridium* also contained 4,4-dimethyl sterols at low levels.

The presence of Δ^7-sterols (both with and without a 4-methyl group) also distinguishes the sterol profile of the rhodophyte from the cryptophytes. Although Δ^7-sterols have been reported in other microalgae they are neither common nor abundant except in some species of chlorophytes (Patterson, 1969). 24-Methylcholesta-7,22-dien-3β-ol is the major sterol in two diatoms (Orcutt and Patterson, 1975) and a chlorophyte (Rezanka et al., 1986), while 24-methylcholest-7-en-3β-ol has been reported in some cyanobacteria, euglenophytes and chlorophytes (Patterson, 1991).

The sterol compositions of the microalgae examined were quite different from macroalgae from the Rhodophyta. Many red macroalgae contain cholesterol as the major sterol, some contain high amounts of stanols and a few contain cholesta-5,22E-dien-3β-ol (Chardon-Loriaux et al., 1976). Species from the Amansia group of the Rhodomelaceae (Rhodophyta) contain a high proportion of 24-methylenecholesterol (Combaut et al., 1981), which was not detected in our work. In contrast, the main sterols of the filamentous red alga *Goniotrichum elegans* (Bangiophyceae) are 24-methylcholesta-5,22-dien-3β-ol and cholest-5-en-3β-ol (Brothers and Dickson, 1980), which is more similar to the cryptomonad compositions (Table 3).

2.4. Phylogeny

The Rhodophyceae are thought to have resulted from a primary endosymbiotic event involving a primitive unicellular photosynthetic cyanobacterium (prokaryote) and a non-photosynthetic protist (Kenyon and Stanier, 1970; Margulis, 1981). Although most prokaryotes do not produce fatty acids more unsaturated than monoenes (Margulis, 1981), photosynthetic filamentous and some unicellular Cyanobacteria synthesise de novo, C_{18} $n-3$ and/or $n-6$ PUFA (Nichols, 1970; Erwin, 1973; Tocher et al., 1998; Sargent et al., 1995; Sperling et al., 2003). The similarity of these "prokaryotic" fatty acid compositions with the compositions of chloroplasts have long been recognised (Kenyon and Stanier, 1970), and since chloroplasts are the main site of $n-3$ PUFA formation from $n-6$ PUFA in photosynthetic organisms (Harwood, 1988; Sargent et al., 1995; Sperling et al., 2003), it is a C_{18} PUFA-producing prokaryotic cyanobacterium which is beleived to have resulted in the chloroplasts of rhodophyte algae (Kenyon and Stanier, 1970; Margulis, 1981).

The candidate hosts for such an endosymbiotic event could have been a non-photosynthetic protist, some of which produce de novo $n-6$ PUFA but not $n-3$ PUFA (Erwin, 1973; Nes and Nes, 1980), and produce the long chain PUFA when the precursor C_{18} $n-3$ and/or $n-6$ PUFA are present in the culture media (e.g. Sul and Erwin, 1997). Thus it was likely the coupling of the chloroplastic desaturase enzyme systems for C_{18} $n-6$ and $n-3$ de novo PUFA production (from a endosymbiotic photosynthetic cyanobacterium, i.e. the primitive chloroplast) to these fatty acid desaturase/elongase enzyme systems of a non-photosynthetic eukaryotic protist, which resulted in the ability of rhodophytes (e.g. *Rhodosorus* sp.) to produce the longer chain and more highly unsaturated C_{20} PUFA such as 20:4$n-6$ and 20:5$n-3$. The enzyme systems derived from the non-photosynthetic eukaryotic protist would include desaturases and elongases of the endoplasmic reticulum (Harwood, 1988; Sargent et al., 1995), as evident from the generally extrachloroplastic C_{20} PUFA production (from C_{18} PUFA) in algae (Khozin-Golderg et al., 2002).

The secondary major endosymbiotic event, (involving a C_{20} PUFA-producing rhodophyte endosymbiont) with a flagellated ancestral protist host (capable of producing the enzymes required for C_{22} PUFA production from C_{20}

PUFA), resulted in cryptomonads able to produce C_{22} PUFA such as 22:5$n-6$ and 22:6$n-3$. The C_{22} PUFA desaturase/elongase enzyme systems derived from the non-photosynthetic eukaryotic protist could include enzymes of the outer mitochondrial membrane (in the case of channelled carnitine-dependent decosahexaenoic synthase –Infante and Huszagh, 1998), or those of peroxisomes (in the case of β-oxidation of 24:6$n-3$, as identified in higher organisms; Voss et al., 1991).

Most rhodophytes (eg. *Porphyridium* spp. and macroalgal rhodophytes) have low proportions of the C_{18} PUFA and high levels of C_{20} PUFA such as 20:4$n-6$ and 20:5$n-3$ (Nichols and Appleby, 1969; Jamieson and Reid, 1972; Cohen, 1990), but *Rhodosorus* sp. and cryptomonads (Table 2, Beach et al., 1970; Renaud et al., 1999) contain relatively high proportions of 18:3$n-3$ and low proportions of 20:4$n-6$ (Table 2). The chloroplast lipids of cryptomonads have high levels of 18:3$n-3$ but only small amounts of $n-6$ PUFA (Beach et al., 1970), which is more similar in composition to *Rhodosorus* sp. (18:3$n-3$ rich, $n-6$ PUFA poor) than to *Porphyridium* spp. or other rhodophytes (18:3$n-3$ poor, $n-6$ PUFA rich) (e.g. Cohen, 1990; Jamieson and Reid, 1972). Similarly, the high level of 24-methylcholesta-5,22E-dien-3β-ol in both *Rhodosorus* sp. and the cryptomonads suggests a close relationship, compared to the very low levels detected in *Porphyridium* spp. (Beastall et al., 1971, 1974). Also Δ^7 sterols are the biosynthetic precursors to the Δ^5 sterols and high abundances of Δ^7 sterols are usually considered the more "primitive" condition (as in *Rhodosorus* sp.) compared to higher levels of Δ^5 sterols in more advanced species (as in the cryptomonads) (Nes and Nes, 1980). However, it is not known why cryptomonads convert all of the Δ^7 sterols to the $\Delta^{5,7}$ sterols and Δ^5 sterols, while rhodophytes do not.

2.5. Application-aquaculture potential of the algal strains

The amino acid compositions of cryptomonads and rhodophyte species were similar to each other, (Table 4) and to microalgae from other classes (prymnesiophytes, eustigmatophytes, diatoms, prasinophytes and chlorophytes) (Brown, 1991). Ten amino acids are thought to be essential for marine animals (Table 4). Although the availability of these essential amino acids was not studied here, the proportions were very similar to proportions in larval oysters (Brown, 1991), which suggests that cryptomonads protein quality is high, at least for these animals.

Most of the cryptomonad strains grown in batch culture had significant concentrations (6–13% of total fatty acids) of 20:5$n-3$ and 22:6$n-3$, the PUFAs considered important for the nutrition of bivalve molluscs (Langdon and Waldock, 1981). The exception was *C. placoidea* which contained only 0.2% 22:6$n-3$ but was a good source of 20:5$n-3$ (11.0%). *Rhodosorus* sp. also lacked 22:6$n-3$ but contained a high proportion of 20:5$n-3$ (20.7%). However, because this alga was difficult to extract, and like other coccoid species such as eustigmatophytes it lacked the C_{22} PUFA, it may prove unsuitable as a source of lipids for the aquaculture industry, as 22:6$n-3$ has been identified as an essential dietary component, especially for fish larvae (Sargent et al., 1995).

The significance of gross composition in determining the nutritional value of an alga may depend on the target species feeding on the alga, and its life stage. For example, high levels of carbohydrate are reported to produce the best growth for juvenile oysters (*Ostrea edulis*; Enright et al., 1986) whereas high dietary protein provided best growth for juvenile Pacific oysters (*C. gigas*; Knuckey et al., 2002). In the present study, our screening of batch cultures of *R. salina* CS-24 and *C. placoidea* showed relatively high proportions of protein compared to most other species, whereas *R. maculata* had one of the highest proportions of carbohydrate. Because of the richness of these three strains in protein or carbohydrate, their distinct PUFA profiles and their good cell division rates, they were tested further in a short-term feeding trial with juvenile Pacific oysters.

The three cryptomonads and the *Isochrysis* sp. (T.ISO) reference diet all produced a greater growth in oysters (as % increase in OW) than unfed oysters (Fig. 1). Oysters fed the microalgae with the highest levels of 22:6$n-3$ (Tables 2 and 5, respectively), namely *R. salina* CS-24 and *Isochrysis* sp. (T.ISO) (60–66% increase in OW) resulted in approximately double the growth of oysters fed the *R. maculata* and *C. placoidea* diets (25–31% increase). The growth rates assessed using changes in DW showed similar trends, though the differences between the diets were not as significant (Fig. 1). *C. placoidea* which produced high 22:5$n-6$ but very little 22:6$n-3$ also resulted in the lowest observed oyster growth rates, suggesting that C_{22} $n-6$ PUFA are not as nutritionally significant as C_{22} $n-3$ PUFA for *C. gigas* spat.

The fatty acid composition of oysters fed on the different diets, as well as starved and commercially reared oysters were also compared, and the composition of the oysters generally reflected that of their diet (Table 5). Whyte et al. (1990) found a similar relationship between the composition of rock scallop (*Crassodoma gigantea*) larvae and its microalgal diet. The proportion of 22:6$n-3$ in *C. placoidea*-fed oysters was ≈60% the value in other microalgal-fed oysters, reflecting the significantly lower concentration of this acid in *C. placoidea.* A similar relationship was seen (though more dramatic) between the concentration of 20:5$n-3$ in *Isochrysis* sp. (T.ISO)-fed oysters and other microalgal-fed oysters, and their diets. The composition of fatty acids in the unfed and commercially grown (i.e. receiving unfiltered, flowing seawater) were very similar. These oysters grew less than the microalgae-fed oysters, yet they contained a higher proportion of 22:6$n-3$ and 20:4$n-6$. *R. salina* produced good growth rates for oyster *O. edulis* spat (Laing and Millican, 1986) and is a popular species in Australian aquaculture. *R. salina* CS-24 has been used successfully as a supplementary food for the commercial-scale production of Pacific oyster spat (McCausland et al., 1999).

Table 4
The amino acid composition (weight %) of the cryptomonads and the rhodophyte

Amino acid (% of total amino acids)	Cryptophyceae							Rhodophyceae
	Rhodomonas sp.	*Rhodomonas/ Proteomonas sp.*	*Proteomonas sulcata*	*Rhodomonas salina* (Wislouch) Hill et Wetherbee	*Rhodomonas salina* (Wislouch) Hill et Wetherbee	*Rhodomonas maculata* Butcher ex Hill et Wetherbee	*Chroomonas placoidea* Butcher	*Rhodosorus* sp.
	CS-215	CS-694	CS-412	CS-174	CS-24	CS-85	CS-200	CS-210
Non-essential								
Alanine	7.7	8.2	7.8	7.9 ± 0.2	8.3	7.9	8.1	8.0
Aspartate	10.3	10.9	10.0	9.5 ± 0.4	9.2	8.9	7.5	9.5
Cystine	0.5	0.5	0.4	0.4	0.5	0.8	0.4	0.5
Glutamate	12.5	11.6	10.6	11.0 ± 0.3	10.7	10.6	10.7	11.1
Glycine	6.1	5.6	6.6	5.6	6.3	5.6	6.4	6.0
Serine	6.5	5.7	8.4	6.0 ± 0.2	6.5	6.1	6.0	6.4
Tyrosine	5.9	6.9	5.7	6.3 ± 0.2	6.1	6.6	5.5	6.2
Essential								
Arginine	5.5	6.3	5.2	5.9 ± 0.2	5.9	5.9	5.2	5.7
Histidine	1.8	2.1	2.7	2.0 ± 0.1	1.9	2.0	1.7	2.0
Isoleucine	4.1	3.7	4.1	4.1	4.3	4.0	4.3	4.1
Leucine	7.6	7.5	7.3	8.1	8.1	8.3	8.2	7.9
Lysine	7.0	7.0	6.6	7.5 ± 0.3	6.3	7.3	6.3	6.9
Methionine	2.1	2.9	2.4	2.5	3.1	2.6	2.7	2.6
Phenylalanine	4.9	4.7	4.5	5.4 ± 0.2	5.1	5.9	4.8	5.1
Proline[a]	4.3	4.2	4.2	5.7 ± 0.2	4.7	4.3	8.5	5.2
Threonine	5.6	5.8	5.4	5.5 ± 0.1	6.0	5.6	5.4	5.6
Tryptophan	2.2	1.5	2.7	1.2 ± 0.1	1.1	2.2	2.1	1.8
Valine	5.3	5.0	5.5	5.4 ± 0.2	5.9	5.5	6.1	5.5

Values from single analyses, except *Rhodomonas salina* CS-24 (duplicate cultures; mean ± range/2).
[a] May be essential for molluscs.

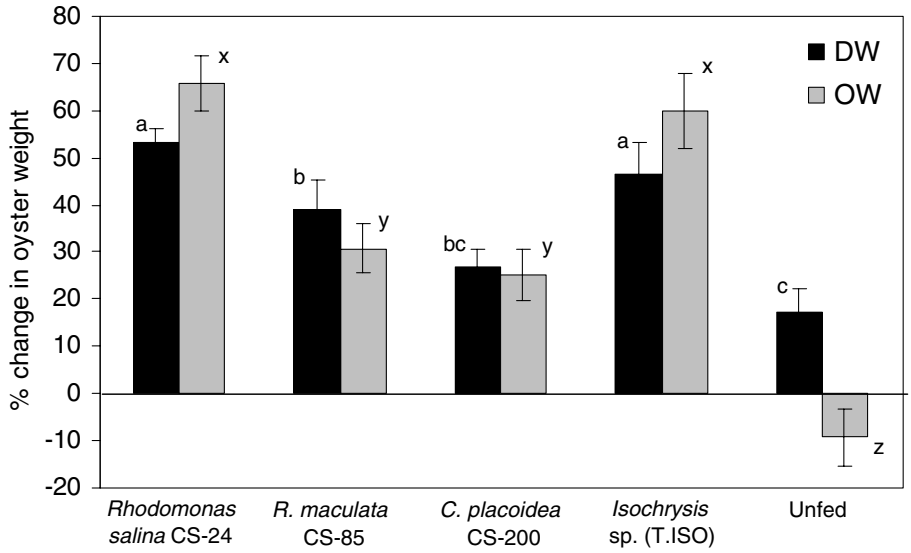

Fig. 1. The percentage increase in dry weight (DW) and organic weight (OW) of juvenile Pacific oysters (initial length 2 mm) fed cryptomonad diets over 2 weeks. Growth is compared to that given by a reference diet, *Isochrysis* sp. (T.ISO), and unfed oysters. Data are mean ± s.d.; bars with a common superscript letter (a–c for DW; x–z for OW) are not significantly different ($P > 0.05$).

Table 5
Relative concentrations of fatty acids in semicontinuous culture of *Isochrysis* sp. (T.ISO) CS-177 (reference diet) and in juvenile Pacific oysters fed for 14 days on *Isochrysis* sp. (T.ISO) CS-177, *Rhodomonas salina* CS-24, *Rhodomonas maculata*, *Chroomonas placoidea* from logarithmic semi-continuous culture

Fatty acid identity or class	Reference diet		Oyster fed corresponding diets				
	Isochyris sp. (T.ISO) CS-177	*Isochyris* sp. (T.ISO) CS-177	*Rhodomonas salina* (Wislouch) Hill et Wetherbee CS-24	*Rhodomonas maculata* Butcher ex Hill et Wetherbee CS-85	*Chroomonas placoidea* Butcher CS-200	Unfed	Commercially grown oysters (wild phytoplankton)
Saturated fatty acids	24.8	22.0	20.9	22.6	21.7	24.3	24.2
Monounsaturated fatty acids	15.6	19.3	14.8	16.3	16.7	18.9	18.5
Polyunsaturated fatty acids	58.5	57.5	63.4	59.9	60.2	53.9	55.5
18:2$n-6$	6.7	4.9	0.7	1.0	5.1	1.1	0.8
18:3$n-6$	2.4	0.9	0.2	0.4	0.3	0.4	0.5
18:3$n-3$	9.8	7.5	11.6	13.0	8.7	2.9	3.1
18:4$n-3$	25.2	17.6	9.6	7.8	6.0	3.6	3.9
20:4$n-6$	–[a]	1.1	1.2	1.8	2.6	3.6	3.4
20:5$n-3$	0.7	3.2	15.9	11.4	13.6	11.1	13.5
22:5$n-6$	1.6	1.8	0.3	0.4	5.6	0.9	0.8
22:6$n-3$	9.6	13.7	14.1	13.3	8.2	18.8	18.0
Branched fatty acids	1.0	1.2	0.9	1.1	1.4	2.8	1.9

The composition of starved oysters and nursery-grown oysters are included for comparison. Data represents mean values from duplicates samples.
[a] Not detected.

3. Experimental

3.1. Microalgal cultures for biochemical screening

Batch cultures of the seven strains of cryptomonad and one strain of rhodophyte were grown in medium f_E. The tropical *Proteomonas sulcata* CS-412 was cultured at 25 °C, the cool temperate *Rhodomonas*/*Proteomonas* CS-694 at 15 °C and the remaining temperate species were grown at 20° (±0.5 °C). Cultures were illuminated from beneath with 70–100 μE m^{-2} s^{-1} white fluorescent light (Philips Daylight tubes) on 12:12 h light:dark cycles in 1.4 L of media in 2 L Erlenmeyer flasks and were aerated with filtered air (0.2 μm membrane filters) supplemented to 0.5% (v/v) with CO_2 at a flow rate of 20 L h^{-1}. To identify analytical variation, two cultures of *R. salina* CS-174 were cultured under identical conditions and analysed. All cultures were harvested towards the end of logarithmic phase; cells were counted with a Neubauer haemocytometer. A cell count could not be determined for the culture of *Rhodosorus* sp. due to excessive cell clumping. Aliquots were collected for the analysis of total lipid, lipid class and fatty

acids (200 mL), protein and amino acids (2 × 25 mL) and total carbohydrate (25 mL).

R. salina CS-24, *R. maculata* and *C. placoidea* were selected on the basis of their composition and cell division rates for testing as food for juvenile Pacific oysters (*C. gigas*). Semi-continuous carboy cultures (10 L) of these strains were established using medium f_E using media and growth conditions identical to that above for the batch cultures. Cultures were harvested semi-continuously during the logarithmic phase by removing approximately 10–20% of the culture volume every day (used for oyster feeding and for dry weight and biochemical analyses) and replenishing the culture with fresh medium. Carboy cultures (10 L) of *Isochrysis* sp. (T.ISO) were also grown under identical conditions except using medium f_2; this alga being used as a reference diet in the feeding trials.

3.2. Total lipid, lipid classes, fatty acid and sterol assays

Cells were harvested from 200 mL of culture medium by filtering through precombusted 47 mm diameter glass fibre filters (GFC) and stored overnight in liquid nitrogen prior to lipid extraction. Samples were extracted with monophasic chloroform–methanol–water (5:10:4, v/v/v, 5 × 5 mL) (modified Bligh and Dyer, 1959). Samples were sonicated between each extraction, and the combined extracts partitioned with chloroform and water to give a final solvent ratio of chloroform–methanol–water (10:10:9, v/v/v). Lipids were recovered from the lower chloroform phase by removing solvents under vacuum and extracts were stored in chloroform under nitrogen at −20 °C. Total lipid was determined gravimetrically on duplicate aliquots.

The proportions of the major lipid classes were determined by analysing a portion of the total lipid extract (in triplicate) on Chromarod S-III silica rods with an Iatroscan Mk III TH-10 TLC-FID analyser (Iatron Laboratories, Japan). The solvent system used was hexane/diethyl ether/acetic acid (60/17/0.2, v/v/v). Lipid classes were quantified using standard curves generated using the same rods and solvent system. The standard solutions analysed in triplicate were five graded concentrations of each of L-α-phosphatidylcholine (polar lipid), 5-cholesterol-3β-ol (sterol), octadeconoic acid (free fatty acid), purified Atlantic salmon oil (triacylglycerol) and purified wax ester from orange roughly oil.

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) under N_2 at 80 °C for 2 h. After cooling and addition of 1 mL of Milli-Q water, the FAME were extracted with 3 × 3 mL hexane–chloroform (4:1, v/v). Solvent was removed with N_2 and the FAME were dissolved in chloroform containing a known amount of methylheptadecanoate as an internal standard. They were then stored at −20° until analysis 1–2 days later.

A subsample of the rhodophyte *Rhodosorus* sp. culture was extracted as detailed above, but none of the pigment (and very little lipid) was removed with this method. A further 60 ml of filtered culture was saponified with 5% KOH (w/v) in methanol:water (4:1, v/v) at 70 °C for 2 h. The pH had dropped to 9 by the end of the reaction. After acidification the fatty acids were extracted with hexane:chloroform (4:1, v/v) and then transesterified as detailed above. This resulted in significantly more FAME (12.3 μg FAME/ml culture medium) than solvent extraction of a duplicate sample (0.15 μg FAME/ml culture medium).

FAME samples were analysed by a Hewlett Packard 5890 gas chromatograph (GC) containing a polar column, and a Shimadzu GC-9A gas chromatograph containing a non-polar column fitted with cool on-column injection ports and flame ionisation detectors (FID). High purity hydrogen was the carrier gas and FID temperatures were at 270 and 310 °C, respectively. Samples (0.4 μl) were injected on a polar 70% cyanopropyl siloxane (BP-X70) fused-silica column (50 m × 0.32 mm i.d.). All samples were also analysed on a non-polar Me-silicone (HP-1) fused-silica capillary column (50 m × 0.32 mm i.d.) to distinguish co-elutions and verify some identifications. Gas chromatograph conditions are detailed elsewhere (Dunstan et al., 1994). FAME standards of known composition were analysed for peak identification and to ensure accurate quantitation. Peak areas were quantified with DAPA integrating software (Kalamunda, Western Australia) on PCs. Fatty acid identifications were confirmed from mass spectra obtained from a Hewlett-Packard 5970 GC-MSD system fitted with a direct capillary inlet; helium was used as the carrier gas. Electron impact mass spectra were acquired and processed with an HP 59970A Computer Work station. Typical MSD operating conditions were: electron multiplier 2000 V; transfer line 310 °C; electron impact energy of 70 eV; 0.8 scans per second; mass range 40–600 Dalton.

Fatty acids are designated as X:Yn-z where "X" is the number of carbon atoms (as 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 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.

For sterol analysis an aliquot of the total solvent extract was evaporated to near dryness under N_2 and saponified in 5% KOH in methanol–water (80:20 v/v) at 80 °C for 2 h. The non-saponifiable lipids (NSL) were extracted with hexane:chloroform (4:1 v/v) and stored under N_2 at −20 °C. This fraction was treated with bis(trimethylsilyl)trifluoroacetamide (BSTFA) immediately before GC analysis to form trimethylsilylether derivatives. The NSL fractions were analysed with a Shimadzu 9A gas chromatograph equipped with an FID and cooled OCI-3 on-column injector (SGE, Australia). Samples in $CHCl_3$ were injected at 45 °C onto a non-polar methyl silicone fused-silica capillary column (HP1, 50 m × 0.32 mm i.d., Hewlett Packard).

After 1 min, the oven temperature was raised to 120 °C at 30 °C/min and then to 320 °C at 4 °C/min. Sterols were identified by gas chromatography-mass spectrometry (HP 5890 GC and 5790 MSD) by comparison of mass spectra with standards or data in the literature.

3.3. Total carbohydrate, protein and amino acid assays

Culture aliquots (25 mL) were filtered through GFC filters. The filters, plus retained algae, were stored for 1–2 months at −20 °C prior to analysis. For carbohydrate analysis, samples were then placed in Mini-vials (5.0 ml; Pierce) together with 4.0 ml of 0.5 M H_2SO_4. The vials were heated at 100 °C for 4 h, then cooled to room temperature and centrifuged (2000*g*, 5 min). Total carbohydrate in duplicate aliquots from the supernatant was determined using the phenol-sulphuric acid colourimetric method (Dubois et al., 1956).

For protein and amino acid analysis, filter samples were placed in vacuum-sealed hydrolysis-tubes containing 2.0 ml of 4 M methane sulphonic acid (with 0.2 μmol nor leucine added as an internal standard and 0.2% tryptamine). Samples were hydrolysed at 110 °C for 24 h. Aliquots (0.4 ml) were removed, and the constituent amino acids purified as outlined in Brown (1991). Amino acids were then derivatised with phenyl isothiocyanate and analysed by reverse-phase high-performance liquid chromatography (Brown, 1991). Total protein content was estimated by summing the anhydroamino acid residues.

3.4. Feeding trial with Pacific oysters

Juvenile Pacific oysters (*C. gigas*) were obtained from Shellfish Culture Ltd, Australia. The oysters were approximately 2 mm diameter shell width and weighed an average of 2.4 mg dry weight (0.18 mg organic weight). They had been hatchery-reared and grown after metamorphosis in a commercial nursery using upwelling systems. Oysters were divided into 17 groups of 100 individuals. Each group was placed in a nylon-meshed chamber contained within a 10 L bucket (McCausland et al., 1999). Buckets were filled with 1 μm filtered seawater (11–13 °C) which was mixed by an aquarium pump (Aquarium PowerHead 480, Second Nature). Three replicate buckets were used for each of three cryptomonad species; *R. salina* CS-24, *R. maculata* and *C. placoidea*; a control group of unfed (starved) oysters (4 replicate buckets) and a reference diet of *Isochrysis* sp. (T.ISO) (4 replicate buckets) were also included. Oysters from all diet treatments were not fed during the first day of the experiment. Thereafter, microalgae were added directly to the buckets at a ration of 8 mg DW bucket^{-1} d^{-1}, this being the satietal intake (i.e. approximately 60–80% of the cells removed d^{-1}) determined immediately preceding the start of the experiment. Through out the experiment, the water in the buckets was changed daily, immediately prior to feeding, and buckets were rinsed free of any waste. The screens were throughly cleaned every second day. This experimental design was adopted to establish the maximum growth rates achievable with the diets. The ration was increased by 20% at the end of the first week (allowing for growth of oysters). After two weeks the growth of oysters was assessed by measuring changes in DW and OW; the latter was calculated as the loss of weight after combusting the oysters for 16 h at 450 °C. Subsamples of oysters were also analysed for fatty acid composition. Data were subjected to an analysis of variance (one-way ANOVA) and by pairwise multiple comparison of means (Fisher's protected least significant difference; PLSD).

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